Tropical *Rhizobium*-legume Symbiosis: Rhizobial Exopolysaccharides and Enzymes.

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You came with me to Britain.
I have achieved my goal leaving you behind.
I only have regrets.

Your silence talks more words than this.
Your smile gives me life.

This is not worth enough to dedicate to you.
However, I offer this to you,
my dear daughter.
And,
I promise not to make you wait near the window
anymore, until I return home after long hours
in the lab.

(PS. *I could not achieve my goal!!!!*)

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Abstract

Although the *Rhizobium*-legume symbiosis has been a widely studied phenomenon in nature, most of the associations which have received much attention were host plants and their symbionts from temperate regions. A wide range of tropical legumes, ranging form herbs to trees, therefore, are yet to be studied. As most of the well-studied *Rhizobium*-legume associations have distinct features which make each association unique, it is reasonable to assume that associations from tropical climates may have new features which are worth study. More significantly, it is known that the nodules of most tropical legumes are different from temperate legume nodules in many aspects, including the influence of rhizobial exopolysaccharides on nodulation. The present work was focused on twelve rhizobial isolates from Sri Lanka, particularly the characteristics of their exopolysaccharides and polysaccharide degrading enzymes and comparison of these with the well-characterised strains, which were mainly from temperate climates.

Research on rhizobial exopolysaccharides has shown that these compounds are necessary for a successful nodulation in many symbiotic associations although the exact role(s) they play is not yet fully understood. The tropical systems studied so far have shown that rhizobial mutants lacking exopolysaccharides also can contribute to the establishment of nitrogen-fixing nodulation. In the present study, the exopolysaccharides of the Sri Lankan rhizobial strains were analysed for their organic substituents, i.e., acetate, pyruvate and succinate groups and main carbohydrate component, i.e., the sugar composition of the polymer main-chains. Chemical methods,

paper chromatography, high performance liquid chromatography, ^{1}H NMR spectroscopy and some enzymological tests were employed to study the structural characteristics of the exopolysaccharides. Most were shown to have considerable similarity to the well characterised polymer, succinoglycan.

In order to understand the extent of the involvement of rhizobial enzymes in symbiosis, the endogenous enzyme activities were examined. Much emphasis was given to find out the presence or absence of depolymerising enzymes acting on plant and rhizobial polysaccharides. In addition to the high level of glycosidase activities which were common for most of the strains, a few had enzymes depolymerising their own exopolysaccharides. Two strains proved unusual in possessing amylase activity.

The biochemical and phenotypic characteristics of the rhizobial isolates used in the present study were also established.

1. Introduction

1.1. Biological nitrogen fixation

The atmosphere contains about 78% of elemental nitrogen, which is a necessary and major component of important organic compounds such as proteins. In common with other elements on earth, nitrogen in the biosphere is involved in a closed cycle in which it is transformed from one chemical form to the other. Neither plants nor animals can use nitrogen in its gaseous form to produce their organic compounds, nor can they transform nitrogen gas into any other form such as ammonia or nitrate which in turn can be utilised as precursors of organic nitrogen compounds. Some conversion of atmospheric nitrogen into nitrates is initiated by the energy given by lightning or UV radiation, but, this is only a very small fraction of the total nitrogen requirement of living organisms. The major conversion (well over 60%) of nitrogen gas into useful forms (ammonia/ nitrate) is done by some procaryotes through the process of biological nitrogen fixation, which involves the enzyme nitrogenase. Procaryotes are the only group of organisms capable of nitrogen fixation and they perform it either as free-living organisms or in symbiosis with plants.

The ability to fix nitrogen is widely distributed among free-living bacteria. *Azotobacter* and *Azospirillum* are examples of free-living, aerobic, nitrogen-

fixers. The nitrogen-fixing organisms *Klebsiella pneumoniae* and *Bacillus polymyxa* are facultative anaerobes. Among the obligate anaerobes, clostridia, methanogens and sulphidogenic bacteria are able to fix nitrogen. Nitrogen fixation by free-living cyanobacteria is widespread in nature. At least forty species of cyanobacteria from different habitats have been identified as nitrogen fixers. Cyanobacteria can also perform as nitrogen-fixers in symbiosis with plants. One of the best known examples for symbiotic cyanobacteria is *Anabaena azollae* which lives symbiotically in the leaf tissue spaces of *Azolla*, a water fern.

The most notable biological nitrogen fixation process in nature can be seen in the symbiotic association between plants of Leguminosae (Fabaceae) and members of the Gram-negative bacterial family Rhizobiaceae, i.e., members of the three genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* which are often collectively called rhizobia. These gram-negative rod-shaped bacteria occur as free-living, strictly aerobic organisms in soil, and, when a host legume is available, establish the symbiotic association. The ability to develop a nitrogen-fixing symbiosis with rhizobia is restricted to legumes, with only one exception, the genus *Parasponia* of the family Ulmaceae (Trinick and Galbraith, 1976). In legume-*Rhizobium* symbiosis, the bacteria and plant together form unique structures called nodules, most often on plant roots (by *Rhizobium* and *Bradyrhizobium*) and sometimes on stems (by *Azorhizobium*) and it is in this nodule that bacteria can reduce nitrogen to

ammonia, which is subsequently assimilated into organic nitrogen compounds. Leguminous plants are very diverse in morphology, habitat and ecology. The family includes about 750 genera and an estimated 16,000 to 19,000 species (Allen and Allen, 1981) ranging from arctic annuals to tropical trees. As the vast majority of these legumes are nodulated by rhizobia, the symbiosis is apparently not an adaptation to a specialised ecological niche but rather depends on some genetic particularity of legumes that is sufficiently complex that it has not evolved anywhere (except *Parasponia*) in the plant kingdom (Young and Johnston, 1989). However, many non-legume plants are capable of establishing nitrogen-fixing nodulation with other bacteria, mainly the species of the actinomycete, *Frankia.*

Although the three rhizobial genera, *Rhizobium, Bradyrhizobium* and *Azorhizobium* have for many years been grouped with the agrobacteria and phyllobacteria into one family, the Rhizobiaceae, modern methods of systematics, such as nucleic acid hybridisation and 16S rRNA analysis have shown the existence of marked genetic diversity within the family (Young *et al.*, 1991). It is now widely accepted that *Rhizobium* and *Bradyrhizobium* are only distantly related (Martinez-Romero, 1994). Each of these genera, especially *Bradyrhizobium*, has more closely related organisms that are not plant symbionts and are placed in different families (Willems and Collins, 1993; Yangi and Yamasato, 1993; Young *et al.*, 1991). Table 1.1 shows some common rhizobial-plant associations. The degree of host specificity varies

largely among the rhizobia (Young and Johnston, 1989). Some strains have a very narrow host range while other strains have a wide range of hosts. For example, *Rhizobium* sp. Strain NGR234 is exceptional in that it nodulates at least 70 different legume genera and the non-legume *Parasponia andersonii* as well (Relic *et al.*, 1994).

Table 1.1. *Rhizobium*-plant associations (van Rhijn and Vanderleyden, 1995)

Bacteria	Host plant(s)
Rhizobium meliloti	*Medicago, Melilotus and Trigonella* spp.
Rhizobium leguminosarum bv. *viciae*	*Pisum, Vicia, Lathyrus* and *Lens* spp.
bv. *trifolii*	*Trifolium* spp.
bv. *phaseoli*	*Phaseolus vulgaris*
Rhizobium loti	*Lotus* spp.
Rhizobium huakuii	*Astragalus sinicus*
Rhizobium ciceri	*Cicer arietinum*
Rhizobium strain NGR234	Tropical legumes and *Parasponia* (non-legume)
Rhizobium tropici	*Phaseolus vulgaris, Leucaena* spp, *Macroptilium* spp.
Rhizobium etili	*Phaseolus vulgaris*
Rhizobium galegae	*Galega officinalis, G. orientalis*
Rhizobium fredii	*Glycine max, G. soja* and other legumes
Bradyrhizobium japonicum	*Glycine max, G. soja* and other legumes
Bradyrhizobium elkanii	*Glycine max, G. soja* and other legumes
Bradyrhizobium st.Parasponia	*Parasponia* spp.
Azorhizobium caulinodans	*Sesbania* spp. (stem nodulating)

As nitrogen gas (dinitrogen) is a highly stable compound, it requires a considerable amount of energy (33.39kJ mol^{-1}) to reduce to ammonia. The chemical process needs elevated temperatures, high pressure and catalysts. However, the nitrogen-fixing microorganisms are able to perform the conversion of nitrogen into ammonia under ordinary conditions using ATP as the energy source. In order to have high ATP production, oxygen is normally needed. However, nitrogenase enzyme is irreversibly damaged if exposed to oxygen. The symbiotic systems between rhizobia and plants have a unique way of solving this problem via a haemprotein called leghaemoglobin. The formation of leghaemoglobin is a specific effect of symbiosis. The protohaem prosthetic group of this compound is synthesised by the bacteria while synthesis of the protein is done by the plant cells. Leghaemoglobin possesses a strong oxygen binding ability and it carries oxygen to the rhizobia in the bound form, so that the oxygen does not damage nitrogenase and is only released to produce ATP through oxidative phosphorylation. Hence, in the symbiosis, the bacterium has the advantage of getting, in addition to nutrients for growth, a sufficient and 'harmless' oxygen supply to produce enough ATP for nitrogen fixation while the plant gets a good supply of fixed nitrogen.

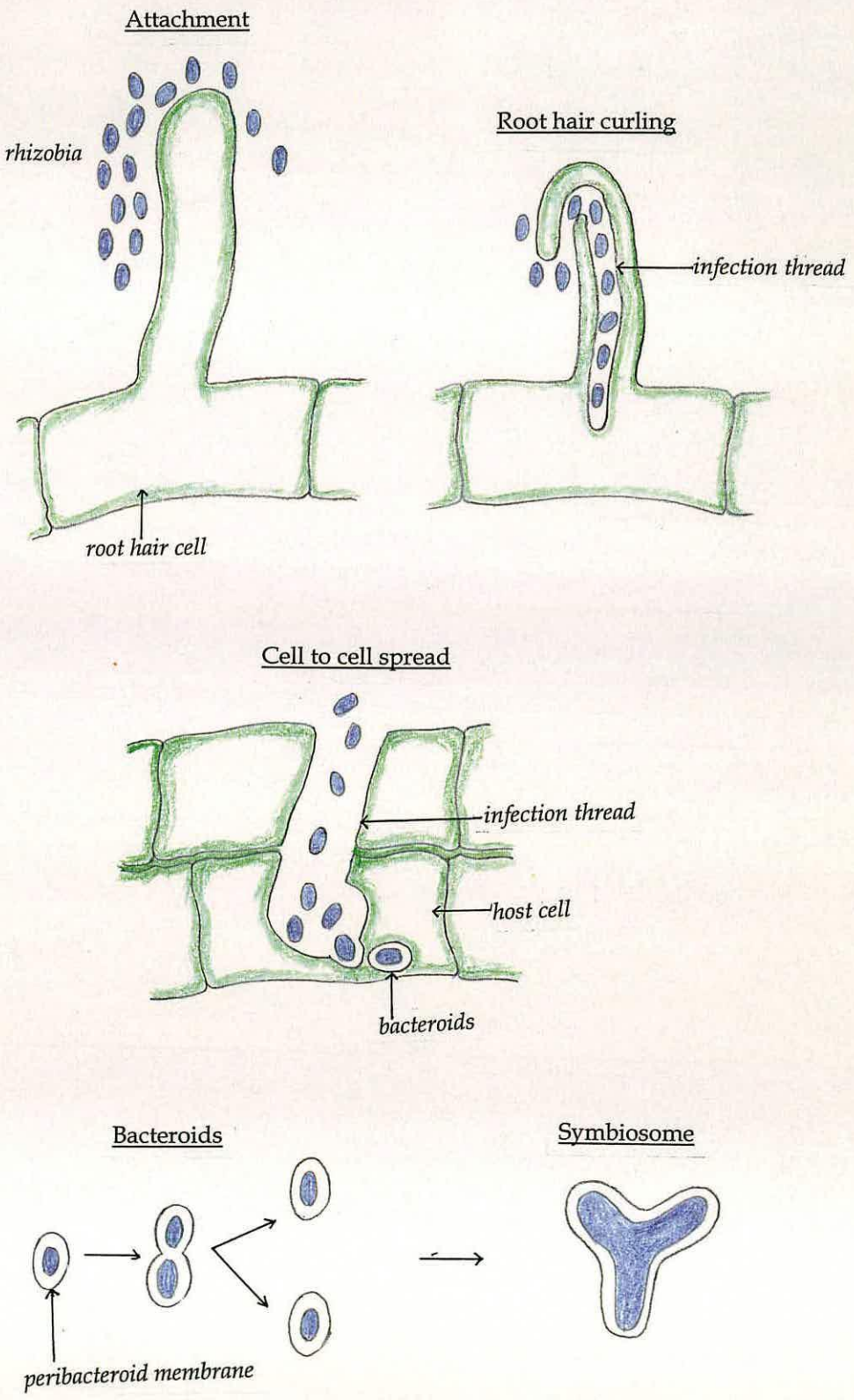
1.2. Developmental biology of the root nodule

The development of the legume-*Rhizobium* root nodule is discussed emphasising the evidence to show the relevance of bacterial cell surface compounds and polysaccharide depolymerases of plant and bacterial origin to the symbiotic association.

1.2.1. General overview of the nodulation process

The visible development of the root nodule begins with the attachment of a compatible *Rhizobium* onto the legume plant root hair. The first structural development is the curling of the root hair. This is followed by the formation of infection threads through which rhizobia invade the root. Even before the infection thread reaches the root cortex, cortical cell divisions are induced. The growing infection threads pass from cell to cell and reach the centre of cortical cell divisions, where, by this time, a nodule meristem is formed. Rhizobia are released from the infection threads into the cells of the evolving nodule. Release of bacterial cells from the infection thread occurs from unwalled regions of infection threads. First, the bacterial cell enters into an outward vesicle of infection thread membrane (plant origin). Then, the bacterial cell, with this membrane envelope, is released from the infection thread into the host cell cytoplasm. This compartment in the nodule cells is called the symbiosome (Figure 1.1).

Fig. 1.1. Stages of development of the root nodule (based on Kannenberg and Brewin, 1994)



Subsequently, rhizobial cell division takes place to form several thousand symbiosomes per nodule cell. The bacterial cells, now called bacteroids, start synthesising nitrogenase which catalyses the reduction of dinitrogen. The membrane of plant origin around each of them is known as the peribacteroid membrane. The ammonia produced by the bacteroids is excreted from the symbiosomes, assimilated by the plant cells and transported to the other parts of the plant. Plants supply bacteria with organic carbon and maintain a low oxygen level around symbiosomes with the help of leghaemoglobin. The tissue containing bacteroids is red because of the presence of leghaemoglobin. The nodules turn green during ageing due to the breakdown of leghaemoglobin into green bile pigments. When the nodule dies, stationary phase rhizobial cells, which are still present in considerable numbers, are released and multiply in soil.

When the significant impact on the plant root structure and function caused by the foreign organism, i.e. rhizobia, is considered, the interactions taking place before the actual nitrogen fixation begins can be generalised as similar interactions to those between plants and pathogenic organisms. Various authors including Vance (1983), Djordjevic *et al.* (1987), Long and Staskawicz (1993) and Downie (1994) compared the properties and activity of rhizobia to those of other plant-invasive microorganisms. Certain pathogenic rhizobia, such as some strains of *Bradyrhizobium* are considered as ancestral forms not yet evolved to symbiosis. Spaink (1995) viewed the development of

symbiotic nodulation as a process in which, to avoid nonsymbiotic infections, the plant uses intricately regulated mechanisms to assess the infection process at various stages and activates resistance strategies if an infection is not moving towards symbiosis. This may also have acted as a strong evolutionary selection pressure towards symbiosis.

1.2.2. Initial interactions

1.2.2.1. Stages of pre-infection

Chemotaxis

Rhizobia are motile bacteria. In *Bradyrhizobium,* the flagellation is polar or subpolar, whereas in *Rhizobium* it is peritrichous (Jordan, 1984). Although non-motile mutants of rhizobia can induce as many nodules on the host plant as the parental strains, the motile cells can have a marked advantage when competing against non-motile mutants (Napoli and Albersheim, 1980; Ames and Bergman, 1981; Mellor *et al.*, 1987; Malek, 1992). However, summarising the results of various authors, Broek and Vanderlayden (1995) considered that *Rhizobium* motility contributes to competitiveness under some environmental conditions but is not of major importance under others.

The roots release various compounds of which some are favourable for rhizobial growth. Both *Bradyrhizobium* and *Rhizobium* are attracted (chemotaxis) by substances including amino acids, dicarboxylic acids,

flavonoids and carbohydrates present in the root exudates. The amino acid homoserine, released by *Pisum sativum* lateral roots stimulates the growth of *Rhizobium leguminosarum*, which results in sufficient bacteria being present to nodulate the plant (van Egerrat, 1975). Since mutants with no flagella produce normal nodulation, chemotaxis is regarded as non-essential in symbiosis. However, the influence of plant exudates helps rhizobial populations to establish in the rhizosphere.

Once a sufficient population of bacteria has been built up, these can begin to influence the plant and possibly infect the host plant to form root nodules. Root nodulation does not normally occur in the field until the first leaves are unfolding and nitrogen fixation does not normally commence until the plant can safely divert a proportion of its photosynthate to this end (Sprent, 1979).

Attachment

After reaching the plant surface, rhizobia attach. Although the primary target site for many rhizobia is the root hair, there is no exclusive site for the attachment. Smit *et al.* (1992) suggested that, in the *Rhizobium*-pea system, it is a two way process which attaches the bacteria to plant root hairs. The first step is an involvement of a bacterial Ca^{2+}-binding protein, rhicadhesin, in direct attachment of bacteria to the surface of the root hair cell. Rhicadhesin is unique to rhizobia and the related genus *Agrobacterium*. Swart *et al.* (1993) showed that rhicadhesin binds non-specifically to many different plants

tested. However, the plant receptor for rhicadhesin or mutants lacking rhicadhesin have not been identified. The other step, tighter adherence, which results mainly in accumulation and anchoring of the bacteria to the surface of the root hair, depending on the growth conditions of the bacteria (such as nutrient limitations), is mediated by plant lectins and/or bacterial appendages such as cellulose fibrils (Smit *et al.*, 1987) and fimbriae (Vesper and Bauer, 1986). Mateos *et al.* (1994) also wrote that extracellular cellulose microfibrils of *Rhizobium leguminosarum* bv. *trifolii* helped the firm attachment of rhizobial cells onto the epidermis of white clover root hair. Initially it was hypothesised that plant lectins play a major role in attachment. Bohlool and Schmidt (1974) and Dazzo and Hubbell (1975) proposed the lectin recognition hypothesis, which stated that lectins with unique sugar-binding properties would attach with specific saccharides on the rhizobial surface. However, Vesper *et al.* (1987) and Loh *et al.* (1993) pointed out that mutants lacking cellulose fibrils or lectins show little or no reduction in their ability to induce nodules. Due to the lack of consistent evidence to prove the lectin hypothesis of attachment and due to the evidence for other mechanisms such as involvement of rhicadhesin, lectin is not now recognised as playing a major role in attachment, other than in the second step described above.

Using a polysaccharide depolymerase isolated from the phage lysate of *Rhizobium trifolii* 4S, Abe *et al.* (1984) fragmented extracellular polysaccharides (and capsular polysaccharides) of *Rhizobium trifolii* 0403 into

oligosaccharides and these oligosaccharides were analysed for clover lectin (trifolin-A)-binding ability and effect on the infection on white clover root hairs. The intact exopolysaccharides did not bind clover lectin but the oligosaccharide fragment did. The oligosaccharides also stimulated infection thread formation in root hairs of clover seedlings inoculated with *Rhizobium trifolii* 0403. This piece of evidence suggests a possible involvement in oligosaccharides originating from exopolysaccharides in this stage of symbiosis and it justifies the need of further studies on exopolysaccharide-degrading enzymes of bacterial, plant, and phage origin.

1.2.2.2. Plant substances inducing rhizobial *nod* genes (*Nod* gene inducers in root exudates)

Some of the root exudates act as inducers of the rhizobial nod genes, of which the products (Nod factors) are essential for the process of nodulation. Flavonoids released by plant roots serve as chemical attractants to *Rhizobium* cells and also induce *Rhizobium nod* genes. Some flavonoids in legume root exudates are, in fact, antimicrobial phytoalexins. For example, coumestrol is released by beans as a response to both pathogenic bacteria and symbiotic rhizobia. However, instead of causing any harm to rhizobia, the flavonoid acts as a *nod* gene inducer (Dakora *et al.*, 1993). Peters *et al.* (1986) reported that the inducer molecule released by alfalfa (*Medicago sativa*) plants for the *nod ABC* gene expression of *Rhizobium meliloti* was luteolin (3',4',5,7-tetrahydroxyflavone), a normal secondary plant metabolite. Phillips *et al.*

(1993) showed that, in addition to luteolin, 4,4'-dihydroxy-2'-methoxy-chalcone, 7,4'-dihydroxyflavone and 7,4'-dihydroxyflavanone also are plant inducers of the *Rhizobium meliloti nod* genes. Naringenin (5,7,4'-trihydroxy-flavanone) from *Vicia sativa* roots is a *nod* gene inducer in *Rhizobium leguminosarum* bv viciae (Zaat *et al.*, 1988) and it was shown to have no *nod* gene inducing ability in *R. meliloti* (Gyorgypal *et al.*, 1991). Fisher and Long (1992) defined the inducers from the plants as phenolics of the flavonoid group, with skeletal or side group modifications to distinguish the inducers from distinct plants.

Several researchers reported the presence of additional flavonoids in root exudates of legumes inoculated with rhizobia, that were not found in exudates of uninoculated plants. Dakora *et al.* (1993) showed that root exudates of alfafa inoculated with *Rhizobium meliloti* contained three isoflavonoids, which were not present in uninoculated plant exudates, namely, the phytoalexin medicarpin, medicarpin-3-*O*-glycoside and formononetin-7-*O*-(6"-*O*-malonylglycoside) which is a conjugated form of the medicarpin precursor formononetin. These authors found that medicarpin does not induce *nod* genes but formononetin-7-*O*-(6"-*O*-malonylglycoside) does and the *nod* gene inducing activity of crude root exudates was increased by 200% upon inoculation with *Rhizobium meliloti.* Agreeing with the results of Dakora *et al.* (1993), Coronado *et al.* (1995) reported results which also suggested that nitrogen-limiting conditions to the plant enhance the

expression of flavonoid biosynthesis genes in the plant and an enhanced production of *nod* gene inducing factors as a consequence.

Spaink (1995) argued that flavonoids may not seem to be likely candidates to function as host-specific signals to *Rhizobium*. First, flavonoids are not unique to legume roots, but are present in several organs of a wide range of plant species. Second, several legume plants exude flavonoids that also activate rhizobia that are unable to nodulate these plants. Nevertheless, Spaink (1995) showed that the results obtained by Horvath *et al.* (1987), Spaink *et al.* (1987), Kondorosi (1992) and Spaink (1994) are strong evidence to regard the recognition of specific flavonoids by rhizobia as an important basis of host specificity. The host specificity of induction process by bacterial signals is determined by the regulatory bacterial NodD protein, which presumably interacts directly with the flavonoids. Spaink (1995) pointed out that the recognition of plant flavonoids by bacterial NodD factors is complex on several grounds. Firstly, all rhizobia recognise broad spectra of (iso)flavonoids as inducers or antiinducers. This ability may reflect an adaptation to the range of compounds produced by different species of host plant. Some rhizobia have a single *nodD* gene that recognise some structural aspects of different flavonoids or have multiple copies of the *nodD* gene with different, more defined, flavonoid specificities (Kondorosi, 1992). Secondly, in the situation when rhizobia have multiple copies of *nodD* genes, there may be a differential response towards certain plant flavonoids. A third

complex feature is that, after induction by bacterial signals, the plant modulates the expression of plant genes involved in (iso)flavonoid biosynthesis

1.2.2.3. *Rhizobium* nodulation genes (*nod* genes) (The genes that control the synthesis of Nod factors)

Fisher and Long (1992) viewed the initial nodulation steps as a two way molecular conversation; the host legume releases signal compounds that stimulate the expression of bacterial *nod* genes. The *nod* genes, in turn, encode enzymes involved in the synthesis of Nod factors that cause morphological changes in plant roots. These Nod factors synthesised by the bacteria are lipochitooligosaccharides, with different host-specific modifications.

The rhizobial genes known as *nod* genes and which are induced by plant substances are located on a large plasmid (*pSym*) in the fast-growing *Rhizobium* species and on the chromosome in the slow-growing *Bradyrhizobium* species. Gottfert (1993) classified *nod* genes into three categories; the common *nod* genes, the host-specific *nod* genes and the regulatory *nod* genes. The common *nod* genes (*nod ABC*) are involved in the synthesis of the lipooligosaccharide backbone of the Nod factors while the host-specific *nod* genes are responsible for the modification of chitooligosaccharide basic structure to make them host-specificity

determinants (Lerouge, 1994). The products of regulatory *nod* genes control the expression of the other two *nod* gene groups upon the interaction with plant factors (Denarie *et al.*, 1992; Gottfert, 1993). The common *nod* genes (*nodABC*) are present in all rhizobial strains and are interchangeable among different species (Kondorosi *et al.*, 1984). The *nod* genes like *nodEF, nodG, nodH* and *nodL* are host specific. Table 1.2 shows the biochemical functions of *nod* gene products. The genes *nod ABC* are essential for nodulation. Another essential gene is *nodD,* of which one or more alleles are present, depending on the rhizobial species. This gene behaves as a common *nod* gene for nodulation in some plants, while in other cases it represents an important determinant of host specificity (cited in Schlaman *et al.*, 1992). In the presence of appropriate plant inducers (flavonoids), rhizobial NodD regulatory proteins (the products of *nodD* genes) activate the transcription of *nod* genes (cited in Denarie and Cullimore, 1993). However, not all the *nod* genes are regulated by *nodD*. Stacey *et al.* (1994) identified the *nodZ* gene of *Bradyrhizobium japonicum,* which is not regulated by *nodD*. The NodD proteins of various rhizobia are functionally different in their responsiveness to different sets of flavonoids and in their *nod* gene activation ability (Horvath *et al.*, 1987, Spaink *et al.*, 1987, Gyorgypal *et al.*, 1991). The structure of a NodD protein determines which flavonoids act as *nod* gene inducers. Therefore, the flavonoid-NodD interactions represent the first major host specific step in the establishment of symbiosis (Schultze *et al.*, 1994).

1.2.2.4. The primary signal for nodule morphogenesis comes from *Rhizobium* (The role of Nod factors)

Both the common and host specific *nod* genes of rhizobia are involved in the production of Nod factors, identified as lipochitooligosaccharides. These compounds are formed by a chito-oligosaccharide backbone consisting of β-(1,4)- linked *N*-acetyl-D-glucosamine with degrees of polymerization ranging from three to five and a fatty acid side-chain at the non-reducing glucosamine residue at the end of the oligosaccharide (Schultze *et al.*, 1992). Modifications to the backbone structure with sulphate (Lerouge *et al.*, 1990; Roche *et al.*, 1991), carbamoyl groups or sugars (Price *et al.*, 1992; Sanjuan *et al.*, 1992; Mergaert *et al.*, 1993), methyl groups (Geelen *et al.*, 1993) and *O*-acetyl groups (Firmin *et al.*, 1993) and the nature of the fatty acyl side chain (Spaink *et al.*, 1991b; Demont *et al.*, 1993) help to determine the host specificity. A single *Rhizobium* species produces a family of structurally related Nod factors varying in the length of the oligosaccharide chain and in the length and degree of saturation of the fatty acyl chain. Different *Rhizobium* species, however, produce Nod factors with characteristic chemical modifications both at the reducing and non-reducing terminus of the oligosaccharide chains (eg. sulphate and *O*-acetyl groups respectively). The Nod factors act as plant morphogens in that they trigger the morphogenic changes necessary for a successful infection and nodule development, namely, deformation and branching of root hairs, formation of

pre-infection threads, cortical cell divisions and formation of nodule meristem.

Depolarization of the cell membrane potential in alfafa root cells due to *Rhizobium meliloti* Nod factor NodRm-IV(C16:2,S) was shown by Ehrhardt *et al.* (1992) as one immediate plant response to Nod factors. Felle *et al.* (1995) studied five different Nod factors displaying different degrees of activity in inducing root hair deformation or cortical cell divisions. They reported that the structural modifications of Nod factors which reduced the activities were correlated with their ability to elicit membrane depolarization, suggesting that it might be a signal which initiates a chain leading to nodulation. However, they pointed out that there may be other different reasons for the observed membrane depolarization.

Table 1.2. Biochemical functions of *nod* gene products of different rhizobial species (Schultze *et al.*, 1994; van Rhijn and Vanderleyden, 1995)

Protein	Function
Synthesis of Nod factor precursors	
NodM (*Rl, Rt, Rm, Bp*)	D-glucosamine synthase.
Synthesis of basic lipooligosaccharide structure	
NodA (Common)	Nod factor acylation.
NodB (Common)	Chitooligosaccharide deacetylase.
NodC (Common)	*N*-Acetylglucosaminyltransferase
Host-specific modifications of Nod factors	
NodE (*Rl, Rt, Rm*)	β-ketoacylsynthase, synthesis of multiunsaturated fatty acid.
NodF (*Rl, Rt, Rm*)	Acyl carrier protein, synthesis of multiunsaturated fatty acid.
NodG(*Rm*)	β-ketoacylreductase, synthesis of multiunsaturated fatty acid?
NodH (*Rm*)	Sulphotransferase, Nod factor sulphation.
NodL (*Rl, Rt, Rm, Bp*)	6-*O*-acetylation of Nod factors at the non-reducing end.
NodPQ (*Rm*)	Sulphate activation.
NodS (*Re, Rtr, Rf.NGR*)	Methyl transferase, *N*-methylation of Nod factors.
NodX (*Rl*)	6-*O*-acetylation of Nod factors at the reducing end.
NodZ (*Bj*)	2-*O*-methylfucosylation of Nod factors.
NolK(*Ac*)	Homology to sugar epimerase, D-arabinosylation of Nod factors?
NolO (*Bj*))	Efficiency of 2-*O*-methylfucosy--lation.

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Table 1.2. (*continued*)

Protein	Function
Auxiliary functions	
NodIJ	Nod factor secretion.
NodN (*Rl, Rt, Rm, Bp*)	Efficiency of Nod signal production.
NodO (*Rl*)	Secreted protein, forms membrane pores.
NodFGH (*Rm*)	Nod factor secretion.
Regulatory functions	
NodD (Common)	Activation of *nod* gene transcription, several alleles in some strains, determinant of host specificity.
SyrM	Flavon-independent *nod* gene activation.
NodVW (*Bj*)	Two-component regulatory system of *nod* gene activation, NodV is the sensor, NodW is the transcriptional activator.
NolA (*Bj*)	*nod* gene regulation?
NolR (*Rm*)	Repression of *nod* gene transcription.

The bacterial strains that produce each Nod protein are given in parenthesis

Rl= Rhizobium leguminosarum bv. *viciae, Rt= R.l.*bv. *trifolii,*
Rp= R.l. bv. *phaseoli, Rm= R. meliloti, Rtr= R. tropici, Re= R. etli, Rf= R. fredii,*
NGR= *R.* strain NGR234, *Bj= Bradyrhizobium japonicum,*
Bp= B strain *parasponia,*
Ac= Agrobacterium caulinodans, Rl= R. leguminosarum.

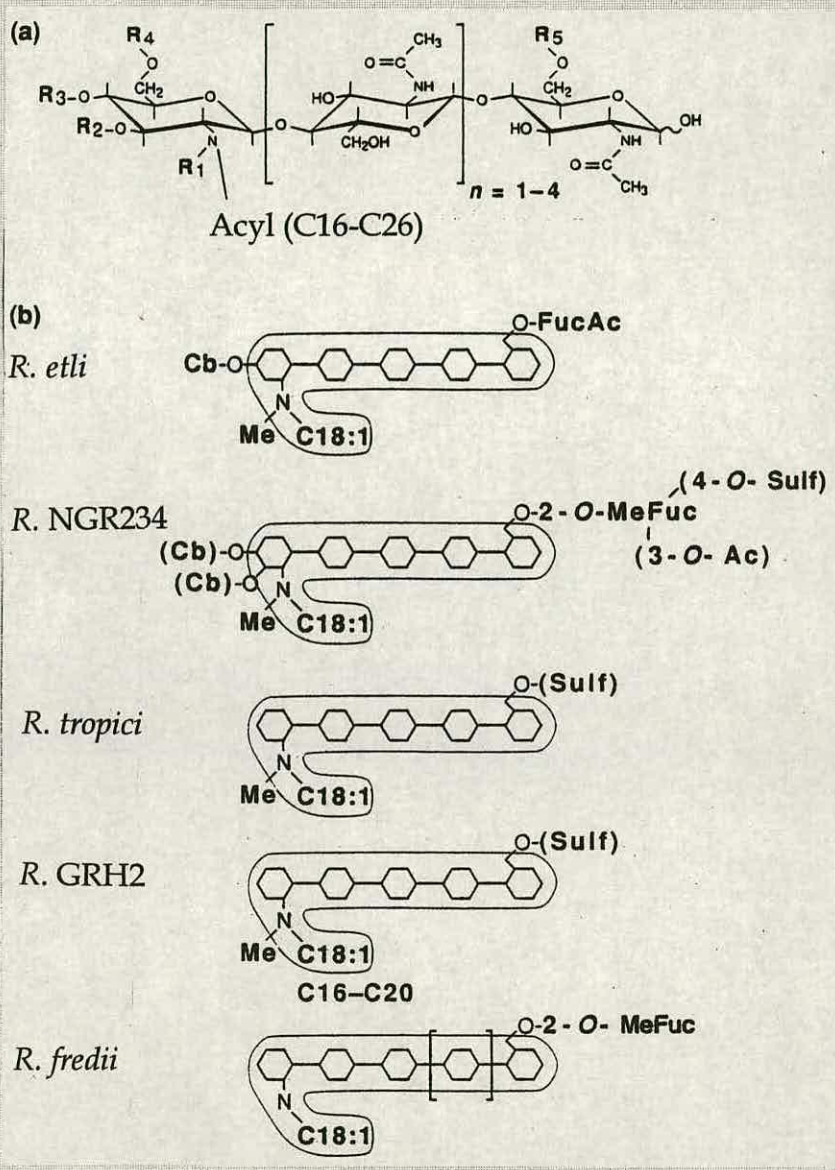
Lerouge *et al.* (1990) identified the major alfalfa specific signal, NodRm-1, of *Rhizobium meliloti* as a sulphated and acylated β-1,4-tetrasaccharide of D-glucosamine. This purified Nod signal specifically elicited root hair

deformation on the host. Truchet *et al.* (1991) reported that NodRm-1 and a related compound Ac-NodRm-1, elicit cortical cell divisions and the formation of genuine nodules on alfalfa seedlings. Chemical modifications of NodRm-1 largely reduced the activity. Non-host plants did not respond to the addition of NodRm-1 at these concentrations. They concluded that a highly specific lipo-oligosaccharide signal is thus necessary to trigger genuine organogenesis in the plant.

Recently, lipo-oligosaccharides from other *Rhizobium* species have been chemically characterised (Spaink *et al.*, 1991a, b). Figure 1.2 shows the basic structure of rhizobial lipo-oligosaccharides *Rhizobium leguminosarum* Nod factors lack sulphate groups and this was the most obvious difference from the *Rhizobium meliloti* Nod factors. A uniform method for naming Nod factors has been proposed by Roche *et al.* (1991), based on various substituents present on the glucosamine backbone. For example, NodRm-1 is now described as NodRm-IV(S). Here, Rm signifies *Rhizobium meliloti,* IV the four glucosamine residues, and S the sulphate on the reducing end of the molecule.

Stacey *et al.* (1994) identified the *nodZ* gene of *Bradyrhizobium japonicum* whose product is required for the addition of a 2-*O*-methylfucose residue to the terminal reducing N-acetylglucosamine of the nodulation signal. This fucosylation was found to be essential for the biological activity of the lipo-oligosaccharide nodulation signal.

Fig. 1.2. General structure of lipo-oligosaccharide Nod factors



(a) General structure of Nod factors

(A backbone of 2-5 β-(1,4)- linked *N*-acetylglucosamine residues linked to one *N*-acylglucosamine moiety at the non-reducing end)

(b) A comparison of Nod factors of rhizobia nodulating *Phaseolus vulgaris*

R_1= methyl (Me), R_2, R_3= carbomyl (Cb), R_4= acetyl (Ac) or Cb
R_5= sulphate (Sulf), acetyl, D-arabinose (D-Ara) or Fucose (Fuc).

R. = *Rhizobium*

Denarie and Cullimore (1993) summarised evidence from various authors which show the various developmental responses in the roots of legumes elicited by purified Nod factors. At nano- to picomolar concentrations, they can induce differentiation of root hair cells and deformation and branching of root hairs (Lerouge *et al.*, 1990; Roche *et al.*, 1991; Spaink *et al.*, 1991b). In the outer and middle cortex, *Rhizobium leguminosarum* factors induce radially aligned cytoplasmic structures believed to be pre-infection threads (van Brussel *et al.*, 1992). In the inner cortex, cells are induced to undergo mitosis, which may be so extensive that it leads to bulging of the root (Truchet *et al.*, 1991; van Brussel *et al.*, 1992). In alfalfa these divisions are often organised, and meristems are formed which occasionally give rise to non-nitrogen-fixing nodules which have the same ontogeny (a cortical origin) and anatomy (peripheral endodermis and vascular bundles) as the bacteria-induced nodules (Truchet *et al.*, 1991)

Staehelin *et al.* (1995) reported a rapid enhanced feedback degradation of Nod factors of *Rhizobium meliloti* after their perception by the host plant alfafa, due to increased activity of plant extracellular enzymes. Their data indicated that perceived Nod factors are inactivated in the rhizosphere within a few hours. They suggested that the rapid inactivation might be an essential step in the establishment of symbiosis by suppressing continous stimulation by active Nod factors which would otherwise lead to inhibitory effects on plant development and to elicit defence reactions. Thus, feedback

regulation of Nod factor molecules would contribute to a process that helps organogenesis while preventing possible defence responses.

1.2.2.5. Nod factors, lipopolysaccharides and bacterial cell membrane

Cedergren *et al.* (1995) in their recent study on rhizobial Nod factor (chitolipooligosaccharide) and lipopolysaccharide (LPS) chemistry uncovered several remarkable common links between the structural chemistry of these two groups of compounds. The authors found that *Rhizobium meliloti* and *Rhizobium* strain NGR234, which are known to possess sulphated chitolipooligosaccharides also have their lipopolysaccharides sulphated. Moreover, a common origin of sulphation was revealed by studying the mutants which were known to be impaired in sulphating their chitolipooligosaccharides. In such cases, there was a corresponding diminution or complete lack of sulphation of lipopolysaccharides. The same structural diversity of the fatty acids in the chitolipoligosaccharides was observed in the membrane lipids. The known functionalization of the chitolipooligosaccharide of *Rhizobium* NGR234 by *O*- and *N*- methylation was also reflected in the LPS of the organism.

The results of Cedergren *et al.* (1995) clearly demonstrated biochemical and spatial links between chitolipooligosaccharides and cell surface lipopoly-saccharides, significant enough to suggest that the Nod factors are, in fact,

membrane components. The authors viewed *nod* genes as genes which encode functions that affect and determine bacterial membrane and general surface chemistry. They concluded that bacterial membrane and cell surface chemistry are critical determinants of the outcome of the symbiotic relationship between rhizobia and legumes.

1.2.2.6. The Nod factor-receptor model for rhizobial invasion (Receptor molecules for Nod factors in the root hair)

The first obvious symptom of nodule formation is the deformation of root hairs. Various suggestions have been put forward to account for the required highly specific interaction between bacteria and root hair surfaces.

The following model has been proposed to describe how the Nod factor induces root hair deformation and cortical cell divisions (Hirsch, 1992).

(1) The N-glucosamine residue of the Nod factor reacts with a sugar-binding site of a receptor in the root hair, presumably a lectin; and

(2) The strength of the interaction between Nod factor and its receptor depends on several properties of the Nod factor: the length of the glucosamine backbone; the presence or absence of various substituents such as sulphate; the composition of the lipid side chain.

Although Nod factor is secreted into the medium by rhizobia, it is proposed to function *in situ* as part of the bacterial membrane with the lipid moiety inserted into the membrane. Two pieces of information support this

proposal: (1) root hair curling factors added to Nod- bacteria do not restore the wild-type conditions (Banfalvi and Kondorosi, 1989; Hirsch, A. unpublished data) (cited in Hirsch, 1992) and, (2) the molecular structure of the Nod factors suggests a membrane location.

The failure of secreted Nod factor molecules to complement Nod- *Rhizobium* may indicate that either (1) a soluble form of the Nod factor is insufficient by itself, or (2) an exact orientation of the Nod factor is essential for the full response of the plant.

The length of the NodRm-1 lipid moiety is estimated to be 2nm (which is approximately equivalent to one-half of a lipid bilayer), strongly suggesting that the lipid tail of the Nod factor is embedded in the rhizobial membrane. Thus, the polar glucosamine head group should extend from the lipid bilayer. Because the Nod factor is a signal molecule, the glucosamine residues are assumed to extend extracellularly.

Receptor molecules that bind the lipo-oligosaccharide (Nod factor) are presumed to be present on the root hairs. The chemical nature of the receptor molecule is so far unknown, but it has been postulated to be a lectin (Lugtenberg *et al.*, 1991). Hirsch (1992) indicated that lectin is the receptor molecule for Nod factors.

The lipo-oligosaccharide factors perceived by the plant initiate a variety of reactions in the root. The elicitation of some of these responses at extremely low concentrations and by factors with very specific molecular structures suggests that high affinity Nod factor receptors are involved. Moreover, the fact that not all the plant reactions have the same Nod factor structural requirement may indicate that more than one type of receptor is involved, perhaps having different affinities for distinct parts of the Nod molecules and/or located in different cell types (Denarie and Cullimore, 1993)

1.2.2.7. *Rhizobium* cell surfaces and initial interactions (The role of surface polysaccharides in the initial interactions)

How is Nod factor related to the lipopolysaccharides, exopolysaccharides and capsular polysaccharides that are associated with bacterial cell surfaces? Hirsch (1992) wrote that the relationship of these cell surface components and Nod factor remains undefined. However, recently Cedergren *et al.* (1995) showed very close biochemical and spatial relationships between Nod factors and lipopolysaccharides (see chapter 1.2.2.5). *Rhizobium meliloti exo* mutants, which induce small, white nodules on alfalfa (Finan *et al.*, 1985; Leigh *et al.*, 1987) have functioning *nod* genes (Klein *et al.*, 1988). They elicit root hair cell deformation and can initiate infection thread formation. However the threads abort in the peripheral cells of the bacteria-free, 'empty' nodule (Finan *et al.*, 1985). Numerous, small vesicles have been observed adjacent to the outer membrane of *Rhizobium meliloti exo* mutants within the aborted

infection thread (Yang *et al.*, 1992). These are not observed in *exo*$^{+}$ bacteria, either because the vesicles are normally masked by the EPS of the wild type *R. meliloti* or because they are produced only by *exo*$^{-}$ bacteria. It is not known whether these vesicles are related to the production of exopolysaccharides or other factors (Hirsch, 1992)

1.2.2.8. Other factors and initial interactions (Bacterial Factor substances)

Hirsch (1992) pointed out that, several factors other than lipo-oligosaccharides influence root hair proliferation, branching or deformation, cortical cell divisions or cause a phenotype known as the thick, short root response- Tsr- (van Brussel *et al.*, 1986). These factors are known as Bacterial factors (BF) and, the structures of some of them have been elucidated. Some factors produced by *Rhizobium leguminosarum* bv. *trifolii* promote root hair proliferation or work synergistically to elicit hair deformation and cortical cell divisions on clover (Hollingsworth *et al.*, 1990). One of these factors, BF-5, which is dependent on nod gene induction by flavonoids, has been identified as *N*-acetyl glutamic acid (Philip-Hollingsworth *et al.*, 1991). BF-5, when added to clover roots, causes root hair branching and tip swelling. It also increases the number of foci of cortical cell divisions. BF-5 does not elicit these responses on alfalfa or *Lotus*.

A factor produced by *Rhizobium meliloti* competes with radio-labelled N-1-(naphthyl)pthalmic acid (NPA) for its binding site (Hirsch *et al.*, unpublished data, cited in Hirsch, 1992). NPA, an auxin transport inhibitor, elicits the formation of nodule-like structures on alfalfa roots; these nodule-like structures contain transcripts for early nodulin genes (Hirsch *et al.*, 1989). This *Rhizobium*-derived factor, which has not yet been identified, is produced by Nod^- as well as Nod^+ strains of *Rhizobium meliloti*. However, luteolin is required for secretion of the NPA-competing factor. Alfalfa roots develop a short root phenotype when treated with a partially purified culture filtrate from *R. meliloti* containing this factor (Hirsch, unpublished data, cited in Hirsch, 1992). It is not known whether the genes required for the production of this factor are essential for the symbiosis.

1.2.2.9. Root hair responses (New proteins produced by the root hair)

Soon after the initial interaction between *Rhizobium* and the host, detectable biochemical changes, in the form of the production of new proteins, occur within root hairs, at least in two plant systems studied, pea and cowpea. These proteins have been called 'nodulins' by some workers and their presence differs in roots inoculated with rhizobia and uninoculated roots. The role of these proteins is unclear (Krause and Broughton, 1992) (cited in Hirsch, 1992).

1.2.3. Formation of the root nodule

1.2.3.1. Infection thread formation

After coming together of bacteria and roots, the next problem is how the bacteria gain entry into the host cells. When compatible bacteria are firmly attached to the root hair, a refractile (or haline) spot becomes visible in the root hair cell wall, which indicates hydrolysis of the cell wall. However, the mechanism of the cell wall hydrolysis is not known. Either the bacteria may induce hydrolytic enzymes responsible for localised cell wall dissolution or the bacteria may exploit plant mechanisms, such as those used when epidermal cells grow out into root hairs (Kijne, 1992). Following dissolution of the cell wall, the cell membrane of the root hair invaginates and cell wall material is deposited around it and the rhizobia within (Callaham and Torrey, 1981). The invagination with the newly formed cell wall forms the infection thread. This consists of one or more rows of proliferating bacteria aligned within the sheath of cell wall origin. The bacteria travel from host cell to host cell via the infection thread and its branches. Once inside the nodule cells, bacteria differentiate into bacteroids, their nitrogen-fixing forms. A number of subcellular changes then take place within the root hair cell, including changes in membrane permeability and cytoskeletal arrangement. Long and Cooper (1988) postulated that each of the above mentioned changes may be a part of or may generate a 'second signal' for inducing cortical cell divisions. Divisions of cortical cells form a nodule primodium,

and the infection thread grows towards this primodium (Libbenga and Harkes, 1973; Newcomb, 1981; Vasse and Truchet, 1984; Wood and Newcomb, 1989). The location of the nodule primodia in the root cortex determines the type of the nodule that develops. This is dependent on the host plant and not on the rhizobial strain (Dart, 1977; Newcomb, 1981).

Most of the tropical legume nodules, such as soybean nodules, have a determinate growth pattern (Newcomb *et al.*, 1979; Turgeon and Bauer, 1985). The nodule meristems formed in the outer cortex of the root and the rhizobia are released into actively dividing cells of meristem (Newcomb *et al.*, 1979; Newcomb, 1981). In this type of nodule, the nodule growth (meristem activity) is restricted to a short period after which the invaded meristematic cells differentiate forming a nitrogen-fixing central tissue in the nodule.

The other type of nodules can be seen in temperate legumes such as vetch, pea and alfafa. Their nodules are cylindrical, have a persistent meristem (Newcomb, 1976), and an indeterminate growth pattern. The meristem, which is formed from inner cortex cells, shows a persistent activity making the nodule elongated (Vasse *et al.*, 1990). While the meristem is still active, rhizobia are released into the cytoplasm of plant cells (Brewin, 1991; Hirsch, 1992; Kijne, 1975; Newcomb, 1981). In this type of nodule, the nodule growth and functioning occur simultaneously. Table 1.3 shows the differences between determinate and indeterminate type of nodules.

Table 1.3. Major differences between determinate and indeterminate nodules (Hirsch, 1992)

	Indeterminate	Determinate
Examples	Alfalfa, pea, vetch,	Soybean, bean, Lotus clover, *Leucaena*
Initial cell division	Inner cortex	Outer cortex
Nodule growth	Cell division	Cell expansion
Effect of *exo* mutant	Fix^-, empty nodules	Fix^+, normal nodules
Effect of *lps* mutant	Fix^- / $^+$, appear normal	Fix^-, abnormal
Infection thread	Broad	Narrow
Origin	Temperate	Tropical/ sub-tropical
Transport	Amides	Ureides
nod gene inducers	Flavones, isoflavanones	Isoflavanones

The formation of infection threads through root hairs is not the only way of entry of rhizobia into roots. In *Arachis hypogaea* (peanut) and *Stylosanthes* spp., the bacteria enter through cracks in the epidermis. In the presence of rhizobia, cortical cell divisions are induced and cause cracks in the epidermis and separation of epidermis and cortex. This makes a passage for the entry and spread of rhizobia while no infection thread is formed. The plant cell divisions result in development of infected central tissue resembling the determinate type of nodules (Chandler, 1978; Chandler *et al.*, 1982; Sprent and de Faria, 1988). *Mimosa scabrella* is infected at the junctions of epidermal

cells, through radial walls, producing indeterminate type of nodules (de Faria *et al.*, 1988).

1.2.3.2. Role of rhizobial cell surface components (The role of the products of the *ndv*, *lps* and *exo* genes)

In the process of the development of symbiosis, rhizobial cells create and occupy a series of specialized niches. At each stage, the cell surface must be adapted to allow the bacterium to survive in the endophytic environment, and also to prevent the host plant from mounting a defence response (Kannenberg and Brewin, 1994). In addition, the different substances of the cell surface, mainly β-(1,2)-glucans, lipopolysaccharides and exopolysaccharides may involve in specific interactions to develop a successful symbiotic association. These cell membrane and cell surface components are encoded by the rhizobial *ndv*, *lps* and *exo* genes respectively.

Cyclic β-(1,2)-glucans (Products of rhizobial *ndv* genes)

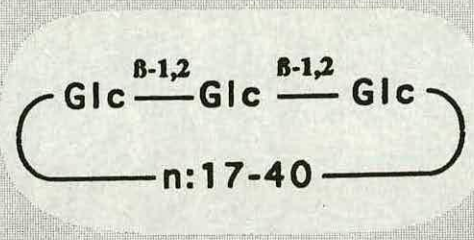
β-(1,2)-glucans are produced by all members of the family *Rhizobiaceae*. These unbranched cyclic molecules (figure 1.3) contain glucose residues linked by β-(1,2)- glycosidic bonds. Their main properties include the β-(1,2)- linked backbone, predominantly 17 to 24 glucose residues, periplasmic localization, osmoregulated biosynthesis with highest level of synthesis occuring during growth at low osmolarity and the presence of phosphoglycerol substituents (Kennedy, 1987; Breedveld and Miller, 1994).

Breedveld and Miller (1995) showed that the transfer of phosphoglycerol moieties to the β-(1,2)-glucan in *Rhizobium meliloti* is inhibited upon an osmotic upshift in the culture medium, however, the biosynthesis was stimulated immediately upon an osmotic downshift.

Rhizobium meliloti ndvA and *ndvB* mutants, which are unable to produce β-(1,2)-glucans, form ineffective white pseudonodules on alfalfa. Closer examination of the pseudonodules reveals a small number of infection threads that abort at an early stage, and no nitrogen-fixing bacteroids. The initiation of infection threads by these mutants suggests that the cyclic β-(1,2)-glucans function during the later stages of alfalfa nodulation (Dylan *et al.*, 1986, Toro and Olivares, 1986; Geremia *et al.*, 1987)

The structures of cyclic β-(1,2)-glucans produced by different rhizobia are very similar. All species of *Rhizobium* and *Agrobacterium* have cyclic β-(1,2)-glucans with their backbone containing 17-21 glucose residues. Thus, as Breedveld and Miller (1994) pointed out, it is unlikely that the cyclic β-(1,2)-glucan backbone confers specificity during nodulation. Instead, it may act as a general signalling molecule. Studies involving *Rhizobium trifolii* and clover (Abe *et al.*, 1982) have shown that exogenously supplied micromolar concentrations of cyclic β-(1,2)-glucans could enhance both nodule number and the kinetics of nodule formation suggesting that the compounds are acting as signalling molecules.

Figure 1.3. Cyclic β-(1,2)-glucan structure



Cyclic β-(1,2)-glucan

Glc = glucose

Additional studies (Dylan *et al.*, 1990b) with *R. meliloti ndv* mutants have revealed that, although the cyclic β-(1,2)-glucans appear to provide important functions during plant infection, these molecules are not essential for nodule formation. It was possible to select for symbiotic pseudorevertants of *ndv* mutants which regain the capacity to form normal nitrogen-fixing nodules on alfalfa yet remain defective for cyclic β-(1,2)-glucan synthesis. It

was noted, however, that the total number of infection threads elicited by such pseudorevertants was substantially smaller than that elicited by wild type cells. Dylan *et al.* (1990b) concluded that the function of β-(1,2)-glucans is likely to be passive and may be involved in the adaptation of the bacterial cell to osmotic variations in its surroundings.

Recently, symbiotic pseudorevertants were closely examined by Nagpal *et al.* (1992). They identified a DNA fragment from an ndvA pseudorevertant gene bank which is able to suppress Ndv phenotype. This fragment was shown to contain several genes involved in the synthesis of succinoglycan (*exo* genes). The mechanism for suppression of Ndv phenotype is unclear, but these researchers speculated that cyclic β-(1,2)-glucans and exopolysaccharides of *R. meliloti* interact during nodule development. Alternatively, they also suggested that the NdvA and NdvB proteins might form a complex with proteins involved in EPS biosynthesis. Both *Rhizobium fredii* and *Bradyrhizobium japonicum* nodulate soybean. *R. fredii* produce cyclic β-(1,2)-glucans, but not *B. japonicum*. Instead, *B. japonicum* synthesize cyclic β-(1,3)-glucans and cyclic β-(1,6)-glucans. Therefore, Breedveld and Miller (1994) suggested that the cyclic nature, not the arrangement of glycosidic linkages, represents the critical feature. This evidence also indicates that the influence of cyclic glucans is non-specific. Cyclic β-(1,2)-glucan biosynthesis continues throughout the nodule development. Rolin *et al.* (1992) and Gore and Miller (1993) showed that *B. japonicum* USDA110 bacteroids in the nodule produce

synthesises only one structural form of its polysaccharides at a time (Gray and Rolfe, 1990)

Dudman (1984) proposed that the uniqueness in structure of the exopolysaccharides and lipopolysaccharides of a *Rhizobium* strain may play a role in its host-specific interaction with plants. There is much evidence available to suggest that the exopolysaccharides have a role in legume-*Rhizobium* symbiotic interactions. Various authors, often using *exo* mutants of *Rhizobium* strains, have demonstrated the lack of some exopolysaccharides affecting negatively on symbiosis, either producing ineffective nodules or producing no nodules at all. However, the specific activities of the exopolysaccharides in legume-*Rhizobium* symbiosis are still largely unknown. Borthakur *et al.* (1986) found that the *exo* mutants of pea and vetch produce no nodules at all, while the results obtained by Diebold and Noel (1989) showed that the nodules formed by the *exo* mutants of the symbiont on clover were not elongated and cell invasion was severely reduced.

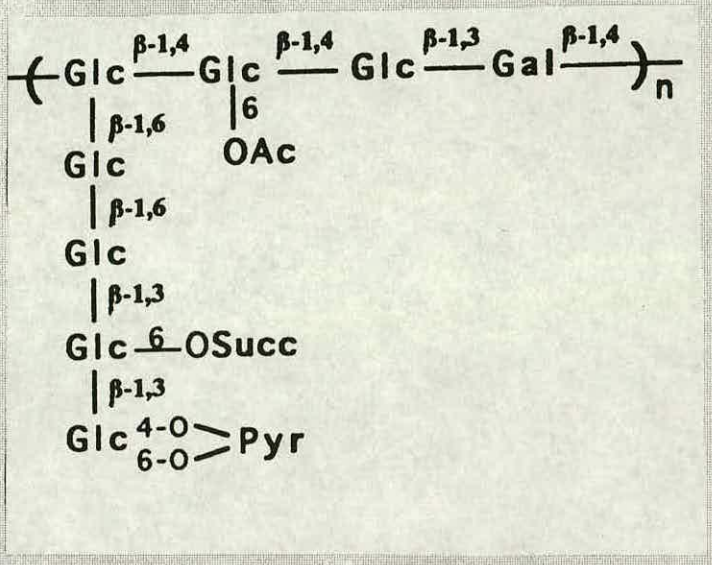
Patseva *et al.* (1991) studied the effect of exopolysaccharides isolated from five *Rhizobium leguminosarum* strains with different degrees of virulence on pea nodulation. When two-day-old pea seedlings were incubated (before being inoculated) with exopolysaccharides for 4h, their nodulation was inhibited whatever the nodulation activity of inoculum strains or the properties of strains producing EPS. The effect exerted by these

exopolysaccharides was different when they were added directly to the inoculum. When EPS from more virulent strains was added to the inocula of less virulent strains, the process of nodulation was always stimulated. On the other hand, the nodulation of otherwise more virulent strains was inhibited by the addition of EPS from less virulent strains.

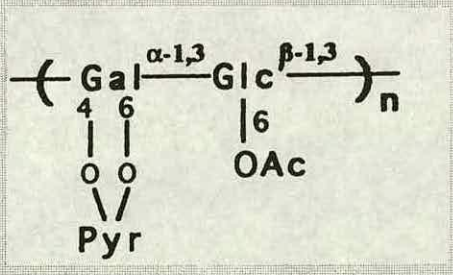
Glazebrook and Walker (1989) showed that *Rhizobium meliloti* strain 1021, which produces succinoglycan, the acidic, calcofluor-binding exopolysaccharide (EPS-I), also had a cryptic capacity to synthesise a structurally different (figure 1.4), non-calcoflour binding, second exopolysaccharide (EPS-II, a galactoglucan), of which the synthesis was found to be induced by a mutation. According to Finan *et al.* (1984) and Leigh *et al.* (1985), Exo$^-$ mutants of *Rhizobium meliloti* fail to synthesise EPS-I and consequently fail to form effective nodules (form ineffective nodules) on alfalfa (*Medicago sativa*). Glazebrook and Walker (1989) found that the production of EPS-II suppressed the defects of Fix$^-$ phenotype of Exo$^-$ mutants on alfalfa, but not on four other host plants that normally are hosts for the strain Rm 1021.

Fig. 1.4. Structures of (a) succinoglycan and (b) galactoglucan of *Rhizobium meliloti*

(a) Succinoglycan



(b) Galactoglucan



Glc= Glucose, Gal= Galactose, OSucc= O-Succinyl group

Pyr= Pyruvate, OAc= O-acetyl group

These observations suggest not only the importance of exopolysaccharides on symbiosis but also that the structural features of bacterial exopolysaccharides are involved in the determination of host range. From these results, it was also pointed out that the effect of exopolysaccharides on host range did not occur at the level of nodule induction, but rather later, at the nodule invasion and development stages (Exo- mutants induce nodulation but only form ineffective nodules).

Reuber *et al.* (1991b) identified 14 *exo* loci that are required for, or affect, the synthesis of succinoglycan (EPS-I) in *Rhizobium meliloti* strain Rm 1021. They reported that the *exo* genes were expressed mostly in the invasion zone and hence suggested that little or no new EPS-I synthesis is needed after nodule formation. [Streeter *et al.* (1992) showed that, in the nodules on soybean formed with *Bradyrhizobium elkanii,* the bacteroids produce nodule polysaccharides which were similar in composition to its EPS. However, *Bradyrhizobium japonicum* produced a totally different polysaccharide in nodules]. The genetic basis of the synthesis of EPS-II was also analysed and strains which produced EPS-I or EPS-II exclusively or neither EPS were constructed. Alfalfa plants inoculated with an EPS-II-producing strain formed nitrogen-fixing nodules, indicating that EPS-II can substitute for the symbiotic role of EPS-I in the nodulation of alfalfa by *Rhizobium meliloti.* However, recently, Becker *et al.* (1993) found that some of the EPS-I defective mutants still produce trace mounts of EPS-I, which might therefore be the

active compound rather than EPS-II. Another interesting discovery about the same symbiotic association was made by Petrovics *et al.* (1993). They reported that the EPS-I can be functionally replaced by a completely different polysaccharide that is rich in 3-keto-3-deoxyoctonate.

The structure of EPS-II, elucidated by Her *et al.* (1990), was a disaccharide repeating unit, β-D-Glc*p*-1,3-α-D-Gal*p*-1,3-, having a 6-*O*-acetyl group attached to most D-glucose residues and a 4,6-*O*-(1-carboxyethylidine) group attached to every D-galactose residue. Reinhold *et al.* (1994) established the detailed structure of symbiotically important succinoglycan (EPS-I) from *Rhizobium meliloti* strain Rm 1021. Previously undetermined locations of the succinyl and the acetyl modifications were determined, in respect to both the residue locations within the octameric oligosaccharide repeating unit and the carbon positions within the pyranose ring.

Reuber and Walker (1993) found a strain of *Rhizobium meliloti* Rm 1021 carrying a mutation in the *exoZ* locus that leads to the production of EPS-I that lacks the acetyl substituent. The *exoZ* mutant nodulated alfalfa normally, indicating that the acetyl substituent is not necessary for alfalfa nodulation by *Rhizobium meliloti* strain Rm 1021. The same study revealed that the succinyl substituent is, however, essential. Mueller *et al.* (1988) showed that a *Rhizobium meliloti* mutant which produced non-pyruvylated succinoglycan failed to invade alfafa roots.

Rhizobium leguminosarum bv. *trifolii* produces an acidic exopolysaccharide without which the bacterium cannot form effective nodules on red clover. Urbanik- Sypnieweka *et al.* (1992) isolated Exo- mutants of *Rhizobium trifolii* 93 and the e*xo* mutation was complemented by the pARF1368 and pARF25 cosmids taken from the gene bank of *Rhizobium trifolii* TA-1 . However, they found that the complementation was not correlated with the restoration of the ability to form effective nodules. Furthermore, when the cosmids were introduced to wild-type *Rhizobium trifolii* 24, it lost its ability to form effective nodules. The exopolysaccharides produced by *Rhizobium trifolii* 93 pAFR1368 and pAFR25, contain less non-carbohydrate residues (acetyl, pyruvyl and 3-hydroxybutanoyl) than the wild type EPS. These data suggest that the biological activity of the particular EPS in symbiosis depends on the content of the non-carbohydrate substituents.

Battisti *et al.* (1992) reported that the invasion of alfalfa by *Rhizobium meliloti* Exo- mutants was restored by the exogenous addition of succinoglycan from *Rhizobium meliloti* wild type. This bacterium produces two forms of succinoglycan (EPS-I): high molecular weight (HMW); and low molecular weight (LMW). The HMW-EPS is a polymer of 10^6-10^7 daltons while LMW-EPS represents monomers, trimers and tetramers of the repeating unit of the polymer. From several forms of LMW succinoglycan differing in repeating subunit multiplicity and charge, a specific oligosaccharide form (the most charged tetrameric form) was found to be active in this regard. Glucksmann

et al. (1993) showed that the *exoG* mutant which mainly synthesizes LMW-EPS could establish nodules. However, the *exoK* mutant, which lacks a β-glucanase that cleaves succinylated EPS into LMW-EPS, was also able to nodulate normally. Kannenberg and Brewin (1994) suggested that, in the case of *exoK* mutant, there may be an alternative way of generating LMW-EPS or that the LMW-EPS might not be essential for nodulation.

Lopez-Lara *et al.* (1993) identified three independent glycosidic structures as exopolysaccharides of wild-type *Rhizobium* sp. (*Acacia*) strain GRH2. One of them was a high molecular weight acidic heteropolysaccharide. A mutant (GRH2-57) that failed to form this acidic exopolysaccharide, formed nitrogen-fixing nodules on *Acacia* plants but infected a smaller portion of cells in the central zone of the nodule (a five-fold reduction in nodule occupancy) than did wild-type GRH2, indicating partial defects.

When the evidence for the ability/inability of the *exo* mutants of rhizobia to form effective nodules on legume plants is summarised, a certain pattern can be identified; *Rhizobium* exopolysaccharides seem to be required for the formation of effective nodules in plants like alfalfa, pea, vetch and clover, i.e., those that form continuously elongating nodules (indeterminate nodules), but not in bean, soybean or *Lotus* in which the round nodules (determinate) are formed.

Kim *et al.*, (1989) studied the symbiotic activity of mutants of *Rhizobium fredii* HH303 defective in acidic EPS synthesis and reported that all the 19 mutants tested were symbiotically effective or partially effective on soybean (a determinate host), indicating that the major bacterial exopolysaccharide may not be required for symbiotic development. Ko and Gayda (1990) investigated the ability to nodulate soybean by different *exo* mutants of *Rhizobium fredii* USDA 191 and reported that a requirement for EPS production is not as critical in the formation of determinate nodules as it is for indeterminate nodules. Hotter and Scott (1991) isolated eight mutants of *Rhizobium loti* PN184, whose parent strain produce an acidic exopolysaccharide. They found that all eight mutants were fully effective when inoculated onto *Lotus pedunculatus,* a determinate nodulating host, but were ineffective when inoculated onto *Leucaena leucocephala,* an indeterminate nodulating host.

Although evidence is available to prove that there is a definite relationship between the EPS requirement and the type of the nodule, this relationship is underscored by instances in which the same strain or species of *Rhizobium* can inoculate plants of both types (Leigh and Coplin, 1992). The basis for the difference in EPS requirement between the two nodule-types of plants is not clear. Stacey *et al.* (1991) pointed out that indeterminate nodules have broader infection threads than determinate nodules, and EPS could form an important component of infection thread matrix (fibrillar interior of the

infection thread) in broad infection threads. However, other evidence suggests that this could not be the only function of EPS in nodule invasion. Bacteria in indeterminate nodules spread into new cells by continued infection thread penetration, while in determinate nodules, bacteria spread primarily by the division of cells already containing bacteria. Leigh and Coplin (1992) suggested that the difference in EPS requirement may reflect the function of EPS in infection thread penetration.

In their review on exopolysaccharides in plant-bacterial interactions, Leigh and Coplin (1992) pointed out that the developmental abnormality of *Rhizobium meliloti exo* mutants on alfalfa is manifested in several morphological and molecular levels (Leigh *et al.*, 1985; 1987; Yang *et al.*, 1992). Some of these abnormalities may be direct consequences of EPS deficiency, while others may represent late consequences of an earlier block in the developmental pathway: root hair curling is delayed; infection threads form but do not penetrate the nodules; structures are observed around bacteria and aborted infection thread that suggest plant defence responses (Yang *et al.*, 1992; Puehler *et al.*,1991; Niehaus *et al.*, 1993); the nodules that form are fully differentiated, but are not elongated and lack a discrete persistent meristem (Yang *et al.*, 1992); once nodules are formed, only 2 nodulins (nodule-specific plant gene products) are found, compared with approximately 18 in normal nodules (Dickstein *et al.*, 1988; Leigh *et al.*, 1987; Norris *et al.*, 1988). These abnormalities are consistent for all exo mutants

tested so far. Although all the defects are consequences of EPS deficiency, it is not known which are the most immediate consequences. Therefore, a step in nodulation that may occur as a direct response to EPS is not yet known.

Callose is a substance produced as a defence mechanism by plants to seal their cell walls after the tissues are wounded, stressed or subjected to invasion by pathogens. Callaham and Torrey (1981) demonstrated that there was no callose deposition at the site of infection thread initiation in symbiosis between *Trifolium repens* and *Rhizobium*. Ahlborn and Werner (1991) tested the effect *in vitro* of bacterial EPS on 1,3-β-glucan synthase which is the plant enzyme responsible for callose synthesis. They reported that the 1,3-β-glucan synthase activity of *Pisum sativum* and *Glycine max* was markedly inhibited by the exopolysaccharides of their symbiotic partners, *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*, respectively.

Leigh and Coplin (1992) pointed out that, although EPS and nodule formation signals (Nod factors) are generated independently, they may work co-operatively during invasion of alfalfa nodules. In co-inoculation experiments, *nod* mutants together with *exo* mutants invaded nodules only when cell-to-cell contacts were allowed (Kapp *et al.*, 1990).

Although the defect in nodule development is generally consistent for all *exo* mutants of a given species on a given plant, it varies with the particular

Rhizobium-plant pair. Hence, the role of EPS might be entirely different in each *Rhizobium*-plant pair. However, it is equally likely that a similar mechanism operates in each case and that the affected stage in nodule development varies (Leigh and Coplin, 1992).

Although no evidence for specific regulation of EPS synthesis by the plant has been obtained, Leigh and Coplin (1992) pointed out that improper regulation or overproduction of EPS synthesis during invasion can be deleterious. Several workers, using EPS overproducing mutants, studied the impact of an overproduction of exopolysaccharides on symbiosis and reported a negative impact. Doherty *et al.* (1988), Zhan and Leigh (1990) and Reed *et al.* (1991) described two mutants of *Rhizobium meliloti*, namely, *exoR95::Tn5* and *exoS96::Tn5*. The *exoR* and *exoS* genes act as negative regulators of EPS synthesis and, therefore, the respective mutants showed an overproduction of EPS. The authors showed the nodulation defects of *exoR* and *exoS* mutants, the former more marked than the latter. They further reported that *exoR*, as a consequence of unlinked suppressors that restored EPS to normal (low) levels, managed to produce nitrogen-fixing nodules. After further studies on *exoR* and *exoS* mutants of *Rhizobium meliloti*, Ozga *et al.* (1994) concluded that the regulation of EPS synthesis is essential for nodule cell invasion and the consequent development of the symbiotic process. In addition to those findings, they showed that when plants were inoculated with a mixture of EPS overproducing mutants (*exoR*) and normal

bacteria, the normal producers dominated and nodulated successfully, indicating the deleterious effect of EPS overproduction is restricted only to the overproducing cells and does not affect the normal producers in the population.

Once nodule development has proceeded past the point of invasion, EPS production does not appear to be necessary in alfalfa (Kapp *et al.*, 1990; Zhan and Leigh, 1990). Indeed, *exo* gene expression seems to decrease after invasion. Although isolated bacteroids exhibited *exo* gene expression (Keller *et al.*, 1988), *in situ* measurements showed that repression of *exo* gene expression occurs after invasion (Reuber *et al.*, 1991a). Table 1.4 shows the rhizobial (and *Agrobacterium*) genes responsible for the synthesis of cell surface components.

Leigh and Coplin (1992) wrote that experimental results have not supported non-specific mechanisms (that do not depend on the particular chemical structure of the EPS) for EPS activities. They did not consider non-specific attachment mechanisms and non-specific passive avoidance of plant defences as roles of EPS, but, pointed out the possibility of a specific role for EPS as an active suppresser of plant defences. Based upon the results of Djordjevic *et al.* (1987); Leigh *et al.* (1987); Muller *et al.* (1988); Battisti *et al.* (1992), Leigh and Coplin (1992) suggested three important points- (a) that the chemical structure of EPS is important in nodule development; (b) that the EPS need

not to be produced by the same bacterial cell that invades; and (c) that EPS is active at a low concentration. They pointed out that a specific signalling role for EPS in nodule development seems likely.

Considering the instances where a particular EPS can be substituted by another EPS (see the results of Glazebrook and Walker, 1989; Zhan *et al.*, 1989) or LPS (Putnoky *et al.*, 1990; Williams *et al.*, 1990), Leigh and Coplin (1992) suggested that a plant (alfalfa) may retain the ability to recognise several different structures at a particular stage in invasion. Also, they wrote that, the three alternate polysaccharides in *Rhizobium meliloti* (succinoglycan, EPS-II and LPS) may have similar three-dimensional structural configurations (Leigh and Coplin, 1992).

Leigh and Coplin (1992) did not completely rule out non-specific mechanisms. They suggested that high molecular weight EPS might influence the efficiency of invasion by providing a morphological component of the infection thread interior. Brewin (1991) also suggested that EPS may play a role as a matrix component of the infection thread. Kannenberg and Brewin (1994) indicated that EPS, by forming a capsule around the cell, may protect the rhizobial cell from recognition by the plant and argued that, if EPS does function as a diffusible signal (as it seems in the cases where exogenously supplied EPS can restore nodulation) it implies that there may be a plant receptor that can recognize a specific EPS macromolecule.

However, they did not exclude the possibility of binding of the exogenously supplied EPS onto the cell surface of the mutant which lacks the EPS, and, thereby, restoring the ability to nodulate.

Table 1.4. ***Rhizobium*** **and** ***Agrobacterium*** **genes responsible for the regulation of the synthesis of cell surface components** (Glucksmann *et al.*, 1993)

Bacterial genes	Function	EPS regulation	β-glucan regulation
Rhizobium meliloti			
ndvA	manufacture	-	
ndvB	export	+	
exoA	glc.2.transferase	-	+
exoB	UDP.gal.epimerase	-	
exoC	phosphoglucomutase	-	-
exoD		variable	+
exoF	galactosyltransferase	-	+
exoG	no polymerisation	-	+
exoH	succinyltransferase	+	+
exoI		+	
exoJ		trace	+
exoK	glucanase (for LMW)	trace	+
exoL	glc.3.transferase	-	+
exoM	glc.4.transferase	-	
exoN	UDP.glc.phosphorylase	trace	
exoO	glc.5.transferase	+	
exoP	chain length determination	-	

continued overleaf

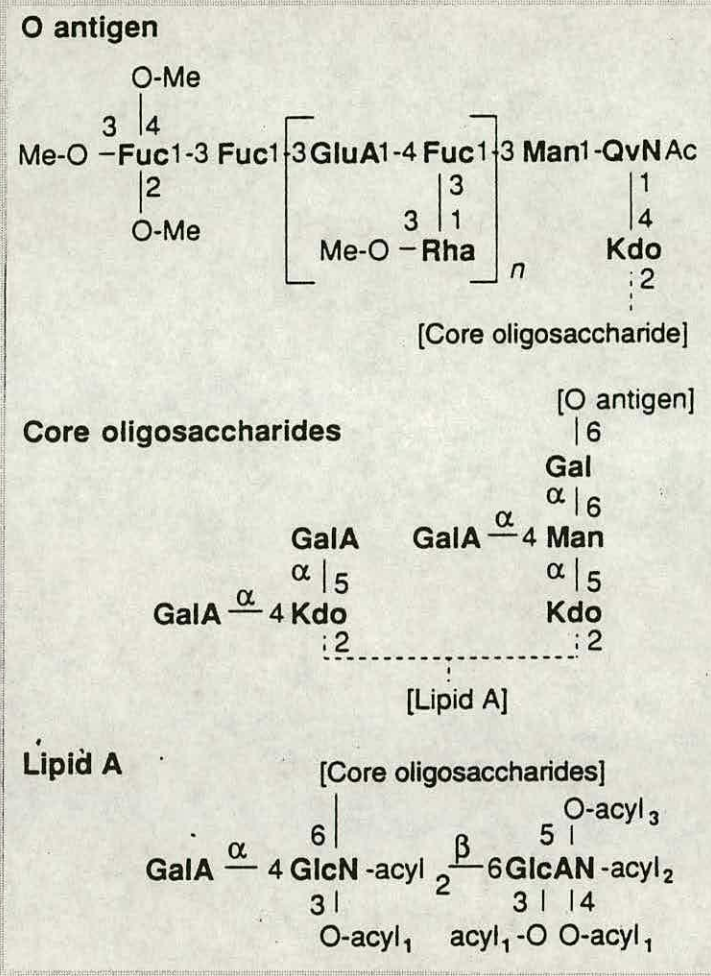
Table 1.4. (*continued*)

Bacterial genes	Function	EPS regulation	β-glucan regulation
exoQ	polymerisation/ export/ transport	-	
exoR			
exoS			
exoT	transporter	-	
exoU	glc.6.transferase	trace	
exoV	pyruvate ketalase		
exoW	glc.7.transferase		
exoX			
exoY	galactosyl transferase	-	
exoZ	acetylase		
Agrobacterium tumefaciens			
chvA		-	
chvB		+	
exoC		-	-

Lipopolysaccharides (Products of rhizobial *lps* genes)

Leigh and Coplin (1992) wrote that while EPS is important in early stages of indeterminate nodule formation, LPS may be important in early stages of determinate nodule formation (Carlson *et al.*, 1987; Cava *et al.*, 1989; Noel *et al.*, 1986 and Stacey *et al.*, 1991). *lps* mutants of indeterminate nodulators are in some cases symbiotically defective as well, but the defect occurred at a later stage of the nodulation process than it does in the case of *exo* mutants (Brink *et al.*, 1990; de Maagd *et al.*, 1989; Priefer, 1989). Lipopolysaccharide (figure 1.5) is one of the major components of bacterial cell outer membrane which creates an interface between the bacterial cell and its environment. Lipopoly- saccharides comprise two classes of macromolecules, LPS-I and LPS-II (Carlson *et al.*, 1992; Raetz, 1993). LPS-I is composed of molecules containing the lipid-A membrane anchor, a core-oligosaccharide and a highly variable and strain- specific polysaccharide, the *O*-antigen. Molecules of LPS-II are basically similar but lack the *O*-antigen. Immunological studies have shown that the external conditions like pH, ion composition and the oxygen concentration can change the LPS structure in free-living cultures of *Rhizobium* (Kannenberg and Brewin, 1989; Kannenberg, 1992; Tao *et al.* 1992). LPS carried by the *Rhizobium leguminosarum* bv. *viciae* in the nodule are different from those of free-living cultures (de Maagd *et al.*, 1989; VandenBosch *et al.*, 1989; Sindhu *et al.*, 1990; Kannenberg *et al.*,1992; Tao *et al.* 1992).

Fig. 1.5. Lipopolysaccharides- General structure



Structure of *Rhizobium* lipopolysaccharide components released after mild acid hydrolysis (Kannenberg and Brewin, 1994).

Gal= galactose, Man= mannose, Fuc= fucose, Rha= rhamnose
GalA= galacturonic acid, GluA= glucuronic acid
QvN= quinovosamine (2-amino-2,6-dideoxyglucose),
GlcAN= 2-amino-2-deoxygluconic acid, Me-O= methyl ester
Ac= N-linked acetate, $acyl_1$= O-linked fatty acids
$acyl_2$= N-linked fatty acids

Mutations leading to an impaired LPS structure (which is similar to the structural changes of LPS under different physiological conditions) in *Rhizobium leguminosarum* lead to either to an arrested infection process (Noel

et al., 1986) or to aberrations in the infection process and in bacteroid development (de Maagd *et al.*, 1989; Priefer, 1989; Kannenberg *et al.*,1992; Perotto *et al.*, 1994).

Observations made by Kannenberg *et al.* (1994) suggest that LPS structural modifications may play an important role in the molecular adaptations of the rhizobial cells to nodule conditions, in particular, in the adaptation of endosymbiotic bacteroids to the peribacteroid fluid. After studying the structural changes of LPS-I through all stages of bacteroid development, the authors concluded that, even after the bacteroids have ceased to grow and divide, structural modifications of LPS are taking place.

After being released from the infection thread, the bacterial cells covered with the envelope from plant cell membrane divide with the envelope giving rise to thousands of symbiosomes; each carry one bacteroid surrounded by peribacteroid membrane. In the case of LPS mutants, the release of bacteria from the infection thread is reduced (Brewin, 1991). According to Perotto *et al.* (1994), LPS mutants, without dividing the peribacteroid membrane in each cell division, accumulate many bacteroids per symbiosome, instead of one as with normal bacteria. This indicates lesser interactions with the peribacteroid membrane by LPS mutants.

Lopez-Lara *et al.* (1995) studied LPS-related aspects of the broad-host-range *Rhizobium* sp. (*Acacia*) strain GRH2, which was originally isolated from root

nodules of *Acacia cyanophylla*. They reported that GRH2-14 and GRH2-50 with mutations that block the biosynthesis of smooth LPS (LPS-II ??) completely prevented bacterial invasion (unlike the EPS mutant GRH2-57 described in the previous section, which was partially defective) in *Acacia* and other hosts of the wild type like *Lotus, Vicia* and *Trifolium* spp. with one exception, *Phaseolus vulgaris*. The EPS mutant earlier described (Lopez-Lara *et al.* 1993) lost the ability to nodulate clover and vetch (indeterminate type nodulation), but retained the ability to nodulate *Phaseolus* and *Lotus* (determinate type), indicating that the importance of EPS is correlated with the type of nodule ontogeny. In contrast, symbiotic involvement of the LPS was not related to the mode of nodule ontogeny, since it was able to nodulate *Phaseolus* and unable to nodulate *Lotus*. In summary, these results showed the importance of EPS and LPS as host range determinants.

1.2.3.3. Other factors involved in nodule development (Possible involvement of plant hormones)

Plant hormones are very important plant substances that play critical roles in different aspects of plant life such as differentiation, development, reproduction, interactions with external factors etc. Schultze *et al.* (1994) pointed out that they are most likely involved in the process of nodule organogenesis too. Though nodule development is a unique programme induced in legume roots by the host specific rhizobial Nod signals, it is likely that all steps in the nodule development pathway are encoded by the plant. To support this hypothesis Schultze *et al.* (1994) showed several findings made by different authors. Under nitrogen starvation conditions, Truchet *et al.* (1989) and Caetano-Anolles and Gresshoff (1992) showed the spontaneous appearence of nodule like structures on *Medicago sativa* in the absence of *Rhizobium meliloti*. Hirsch *et al.* (1989) found that nodule like structures could be induced on *Medicago sativa* in the absence of *Rhizobium meliloti* by polar auxin transport inhibitors. These data indicate that altered hormone levels, as a consequence of nitrogen limitation or transport inhibitors, might provide signals for nodule organogenesis (Schultze *et al.*, 1994).

The ontogeny, morphogenesis and histological organization of spontaneous nodules of *Medicago sativa* have been studied in detail (Joshi *et al.*, 1991) and are similar to those of *Rhizobium*-induced nodules (Newcomb, 1981). Histological observations showed that these spontaneous nodules had

characteristic features of indeterminate nodules such as an active meristem, cortex, vascular strands, central zone with parenchyma cells, endodermis and the presence of large and smaller cells. There were no bacteria or infection threads (Joshi *et al.*, 1991). Blauenfeldt *et al.* (1994) reported spontaneous nodule development on the roots of white clover under nitrogen starvation and in the absence of *Rhizobium.* The structure of these nodules was similar to that of *Medicago sativa.*

EPS-deficient *Rhizobium leguminosarum* bv. *viciae* mutants are unable to nodulate *Vicia sativa* spp. *nigra* (vetch) and *Pisum sativum.* van Workum *et al.* (1995) discovered a correlation between ethylene production (resulting from rhizobial inoculation) by the host plant root and the nodulation ability of EPS-deficient rhizobia. Using conditions to suppress the ethylene production (inhibitors and darkness) in vetch plants, they found that EPS-deficient mutants of *Rhizobium leguminosarum* were able to nodulate when the ethylene formation was minimized. Based on their findings, van Workum *et al.* (1995) suggested the following hypothesis: Nod factors of the rhizobia induce formation of ethylene in the host plant, which, by influencing root growth, inhibits proper root infection by rhizobia. However, in the case of delayed nodulation caused by EPS deficiency, ethylene formation precedes root infection and nodulation is impaired. If root infection is in time (in the case of normal EPS-producing rhizobia), the amount of ethylene is sufficiently small to allow a proper infection thread formation.

Schultze *et al.* (1994) wrote that the most crucial step in the nodule initiation, in which hormones are likely to be involved, is the activation of cell division cycle in the differentiated cortical cells of the emerging root hair zone of the root. Truchet *et al.* (1991) reported the formation of meristematic foci and the subsequent development of empty nodules on *Medicago* roots just by the treatment of purified Nod factors of *Rhizobium meiloti*. This indicated the possibility of a change in the plant root hormonal balance (auxins, cytokinins), resulting in organogenesis, by the Nod factor. Since purified Nod factor alone managed to cause the complete organogenesis, additional compounds produced by the bacteria, including hormones, can be excluded as necessary compounds for nodule formation. However, in some other *Rhizobium*-legume systems, as shown by Spaink *et al.* (1991), Carlson *et al.* (1993) and Mergaert *et al.* (1993), purified Nod factors are capable of initiating the cortical cell divisions and nodule primodia, but unable or only rarely cause the full development of nodules, indicating that the involvement of other compounds, perhaps bacterial hormones, cannot be excluded.

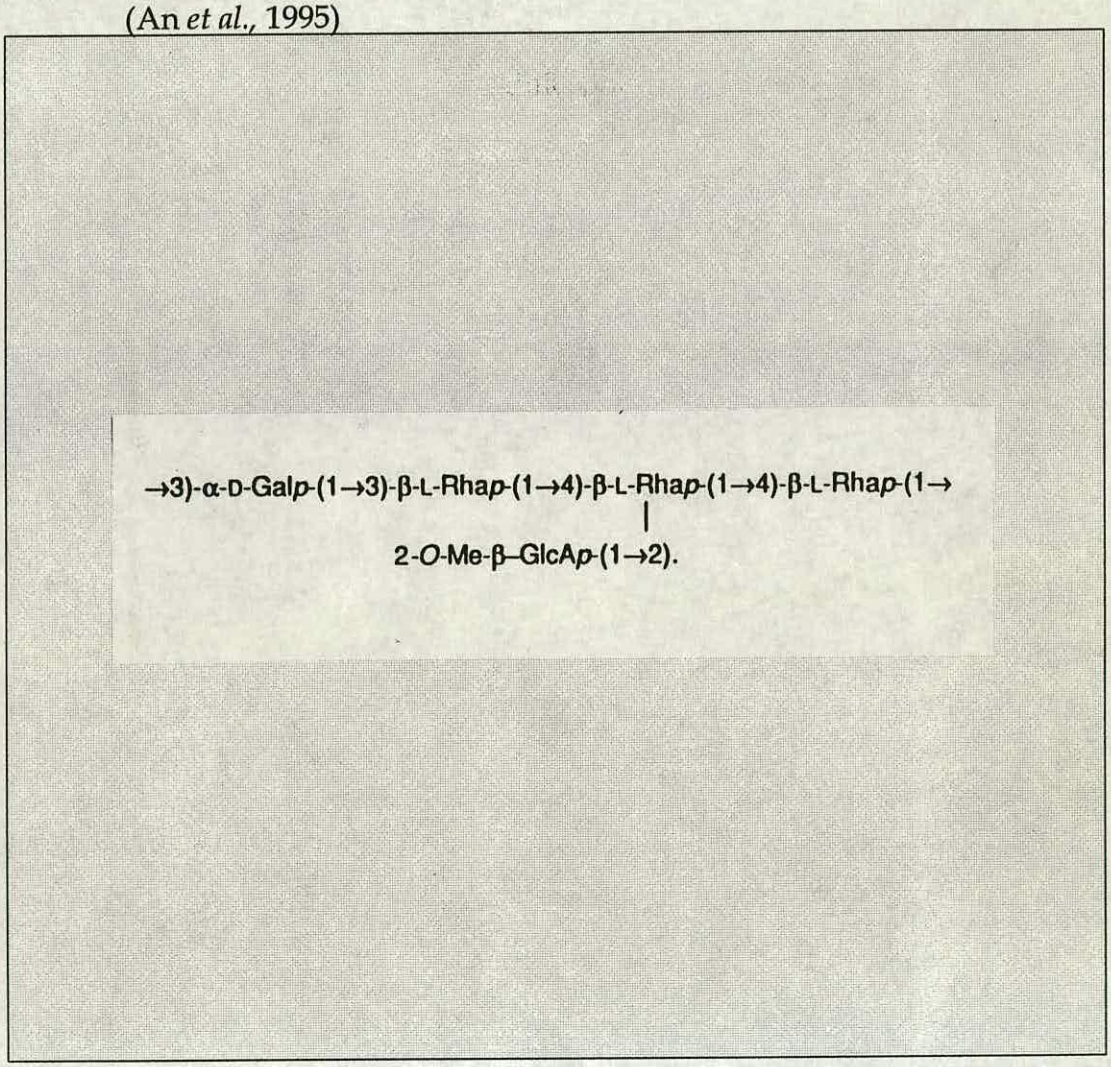
1.2.4. The life of the nodule: some significant features (Nodule polysaccharides, carbon metabolism, peribacteroid membrane)

1.2.4.1. Bacterial nodule polysaccharides (NPS)

Certain strains of *Bradyrhizobium japonicum* and *B. elkanii* (previously called type II *B. japonicum*) synthesise large amounts of polysaccharides in root nodules (nodule exopolysaccharides-NPS) of *Glycine max* L. Merr. (Soybean) (Streeter *et al.*, 1992). For *Bradyrhizobium japonicum,* the composition of NPS is very different from that of its EPS produced in culture, indicating that genes responsible for NPS synthesis are induced in the nodule environment. EPS of *Bradyrhizobium japonicum* contains glucose, mannose, galactose, galacturonic acid and 4-*O*-methylgalactose (Mort and Bauer, 1982; Mort, 1983), while the NPS is composed of galactose, rhamnose and 2-*O*-methylglucuronic acid (Streeter *et al.*, 1992). However, in the case of *B. elkanii,* Streeter *et al.* (1992) found that the NPS composition (rhamnose and 4-*O*-methylglucuronic acid in 3:1 ratio) is identical to that of its EPS which was already elucidated by Dudman (1978). An *et al.* (1995) established the NPS structures (figure 1.6) of both *Bradyrhizobium japonicum* and *B. elkanii* and reported that both NPSs are rich in rhamnose and contain a methylated glucuronosyl residue. They suggested the general NPS structural motif might have an important function in nodule physiology. As the strains of bacteria which produce NPS are the most common within the serogroups known to be dominant for nodule formation in the field, Streeter *et al.* (1992) suggested that NPS may

provide some advantage to nodule occupants. In an attempt to understand the impact of the deposition of nodule polysaccharides on nodule function, Streeter and Saliminen (1993) observed that NPS accumulation in nodules does not interfere significantly with nitrogen fixation per plant, at least for a period of about 55 days after planting.

Fig. 1.6. ***Bradyrhizobium*- Nodule polysaccharide structure**
(An *et al.*, 1995)



1.2.4.2. Carbon metabolism

Various authors including Havelka and Hardly (1976), Ryle *et al.* (1979a) and Ryle *et al.* (1979b) showed that the nitrogen fixation by *Rhizobium*-legume symbiosis is dependent upon the photosynthate transported to the root nodules. Bach *et al.* (1958), Pate (1962) and Reibach and Streeter (1983) showed that sucrose and glucose are the primary compounds transported to the root nodules. However, organic acids like succinate, malate and fumarate are the preferred substrates of the bacteroids (Bergerson and Turner, 1967). The active uptake of organic acids by rhizobia, both in culture and in symbiosis, was shown by San Francisco and Jacobson (1985). Finan *et al.* (1983) showed that the mutants which were unable to take up succinate only formed ineffective nodules. Ineffective nodules have also been induced by dicarboxylic acid transport mutants in *Rhizobium meliloti* (Bolton *et al.*, 1986) and *R. trifolii* (Ronson *et al.*, 1981). El-Din (1991) reported the formation of ineffective nodules on soybean by a succinate transport mutant of *Bradyrhizobium japonicum.*

Indeterminate types of nodules usually export fixed nitrogen as amides. Plants having determinate type of nodules have uride exporting nodules. Studies by Day and Copeland (1991), Streeter (1991), Vance and Heichel (1991) and Gordon (1992) described one of the important aspects of symbiosis, i.e. provision of carbon substrates by the legume host to support nitrogen fixation in the nodule. They reported that, in the determinate

nodules, sucrose is converted in the host cytosol to organic acids, which are the preferred substrates taken up by the bacteroids. Copeland *et al.* (1995) studied the carbon metabolism taking place in indeterminate type chickpea (*Cicer arietinum* L. cv. Amethyst) nodules. They reported that all the capacity for sucrose catabolism was in the host cytosol of the nodules, while the bacteroids lack the key enzymes required. A similar lack of capacity for sucrose catabolism was reported in bacteroids of soybean nodules formed with *Bradyrhizobium japonicum* (Copeland *et al.*, 1989)

Bradyrhizobium japonicum bacteroids accumulate poly-β-hydroxybutyric acid (PHB) during symbiosis at the same time that they fix nitrogen (Mcdermott *et al.*, 1989), and the bacteroids incubated in low levels of oxygen can utilize PHB as a source of energy and reductive power for nitrogen fixation (Bergerson and Turner, 1990). It has also been proposed that PHB accumulation functions as a sink for reductive power, and, by sequestering reduced nucleotides, allows the tricarboxylic acid cycle to operate microaerobically (Mcdermott *et al.*, 1989). Nevertheless, it is not known if free-living *Rhizobium* species operate as microaerophilic organisms that are partially engaged in fermentation.

Encarnacion *et al.* (1993) and Encarnacion *et al.* (1995) reported a fermentation-like metabolism of several strains of *Rhizobium*, including *Rhizobium etli* CE3, when grown in minimal media. This metabolism was

characterized by the excretion of organic and amino acids into the medium and PHB accumulation. As PHB is also a fermentative product and excretion of organic and amino acids are fermentative responses, the authors suggested the term "fermentation-like metabolism" to describe the process. Furthermore, they showed that the addition of compounds to the minimal medium that affect substrate concentrations and/or enzyme activities within or auxiliary to the tricarboxylic acid cycle, prevented the fermentation-like metabolism.

1.2.4.3. Peribacteroid membrane

Peribacteroid membrane is a new form of plant membrane seen in the nodulation process. It originates during the internalization of symbiotic bacteria into plant cells. During the symbiotic interaction, the structure and function of the peribacteroid membrane gradually differentiate from those of the plant cell membrane. For instance, it is no longer involved in synthesis of cell wall components (Rae *et al.*, 1992). It possesses specific transport systems to regulate the exchange of metabolites between the host cell and the bacteroids (Day and Copeland, 1991; Rosendahl *et al.*, 1992; Day and Udvardi, 1993). In addition to some nodule specific proteins (nodulins) targeted to the peribacteroid membrane (Miao *et al.*, 1992), other components of the peribacteroid membrane are characteristic also to the plasma membrane, endoplasmic reticulum and tonoplast of the plant cells. Individual host cells in the central tissue of a nodule contain several

thousand bacteroids, so that the surface area of PMB is 30-100 fold greater than that of the plant cell membrane (Brewin, 1990; Verma *et al.*, 1992)

1.3. Exopolysaccharides: physico-chemical features

The term exopolysaccharides (EPS) is used to cover polysaccharides found external to the structural outer surface of the microbial cell and these may either be associated with outer surface macromolecules or totally dissociated from the cell. EPS may form a part of a capsule firmly attached to the cell surface or may be observed as loose slime not directly attached to the surface. They show considerable diversity in their chemical composition and structure. EPS are mainly composed of carbohydrates; in addition, there may be organic and inorganic substituents. The carbohydrate components are extremely diverse. They are often common monosaccharides like D-glucose and D-galactose in the pyranose forms. In addition some may be rare sugars like L-hexoses or furanose forms of the hexoses, glucose and galactose. There are also various amino sugars. The presence of uronic acids such as D-glucuronic acid is common and as a result many exopolysaccharides are polyanionic in nature.

The most common organic substituents found in microbial exopolysaccharides are ester-linked acetates and pyruvate ketals. Succinyl groups are sometimes found along with acetyl groups. Another ester-linked substituent which may be present is 3-hydroxybutanoate. A common

inorganic substituent is phosphate. It was long thought that sulphate was limited to eukaryotic polysaccharides and proteoglycans, but it now appears that sulphate is present in some prokaryotic polymers. As yet, these are confined to species of Cyanobacteria and Archaeobacteria (Sutherland, 1985; 1988; 1990; Parolis *et al.*, 1996). Sutherland (1990) pointed out that many of the descriptive reports on extracellular polysaccharides in the laboratory and in the natural environments have failed to recognise the relationship between the physical forms of these macromolecules and the physiological conditions present. It was further noted that changes in the growth conditions can drastically alter the composition, physical properties and organisation of the polysaccharides at the bacterial surface.

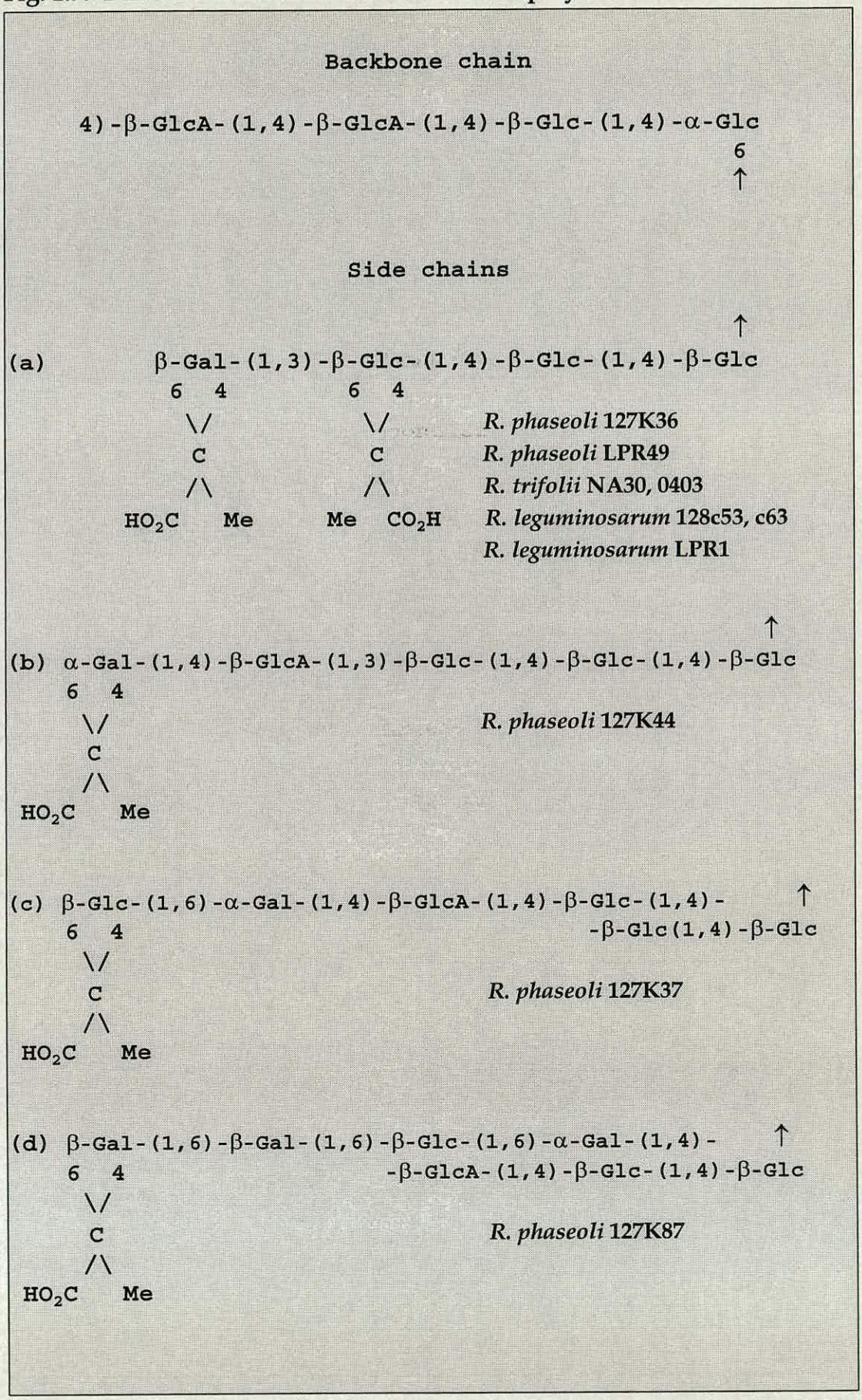
Table 1.5. Carbohydrate component in microbial exopolysaccharides (Sutherland, 1990)

Common monosaccharides	
D-glucose (pyranose)	present in
D-galactose (pyranose)	many microbial
D-mannose (pyranose)	exopolysaccharides.
6-deoxyhexoses	
L-fucose	frequently present
L-rhamnose	frequently present
Pentoses	
D-ribose	Cyanobacteria
D-xylose	Cyanobacteria
Rare sugars	
L-hexoses	present in some EPS.
glucose (furanose)	present in some EPS.
galactose (furanose)	present in some EPS.
N-acetyl amino sugars	in some species, e.g. *E. coli*
N-acetyl-D-glucosamine	commonest amino sugars
N-acetyl-D-galactosamine	commonest amino sugars
N-acetyl-D-mannosamine	occasionally found
N-acetyl-D-fucosamine	rare
N-acetyl-D-talosamine	rare
Uronic acids	present in many EPS
D-glucuronic acid	commonest
D-galacturonic acid	less common
D-mannuronic acid	in small number of EPS
L-iduronic acid	*Butyrivibrio fibrisolvens*
Hexosaminouronic acids	in wide range of EPS

Rhizobium trifolii has the ability to produce three different types of polysaccharides, namely, acidic EPS, neutral insoluble capsular polysaccharide and cyclic-β-1,2-glucans. Zevenhuizen (1986) observed the production of EPS, capsular polysaccharides and cyclic-β-(1,2)-glucans by *Rhizobium* trifolii strain TA-1, under different batch culture conditions. Under air saturation, the major polysaccharide produced was acidic exopolysaccharide. Under a moderate oxygen supply and nitrogen limitation, the neutral insoluble capsule polysaccharide was the major product, while cyclic-β-(1,2)-glucans were dominant at high cell density and severe oxygen limitation.

Rhizobium meliloti SU47 was known to produce its second exopolysaccharide, (EPS-II), only in strains that were genetically altered to activate EPS-II synthesis. Zhan *et al.* (1991) showed that the ability to produce EPS-II is not entirely cryptic, but occurs under conditions of limiting phosphate. They reported the EPS-II production by *exo* mutants of *Rhizobium meliloti,* under a relatively restricted set of environmental conditions. Zevenhuizen and Faleschini (1991) studied the EPS synthesis of *Rhizobium meliloti* mutant strain YE-2, which produces both succinoglycan (EPS-I) and galactoglucan (EPS-II), under different osmolarity conditions. They found that the osmotic conditions of the culture medium markedly affect the production of EPS. At low osmolarities (with no added NaCl), the mixture of EPS comprised

Fig. 1.7. Different structures of *Rhizobium* exopolysaccharides



approximately 75% of galactoglucan and 25% of succinoglycan. When the osmolarity was increased by addition of NaCl until 0.6M, the succinoglycan proportion increased up to 80%.

When the large number of microbial exopolysaccharides is considered, the number of structures which have been studied in detail is still relatively small. Microbial exopolysaccharides show a high degree of diversity in their function and some of the functions are not yet clearly understood. One of the areas of particular interest is the role of exopolysaccharides of *Rhizobium* in legume-*Rhizobium* symbiosis.

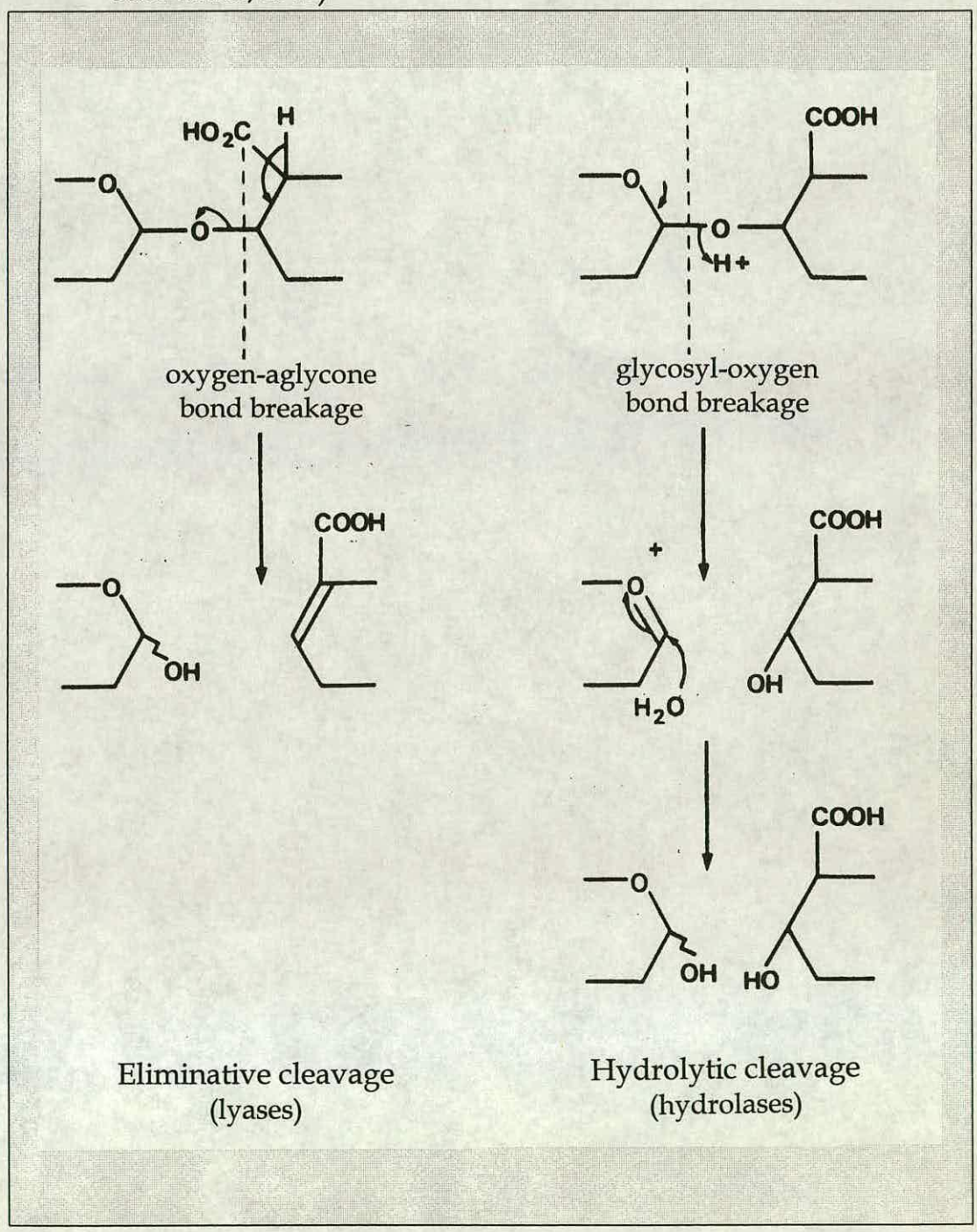
1.4. Depolymerase enzymes

1.4.1. Exopolysaccharide depolymerases

There are two main types of exopolysaccharide-degrading enzymes, namely, hydrolytic enzymes (polysaccharases) and lyases (eliminases) and these enzymes show a very high degree of substrate specificity. Hydrolases and lyases have different mechanisms of action; hydrolytic cleavage and eliminative cleavage (figure 1.8). In hydrolysis, the glycosyl-oxygen bond in the glycosidic linkages of the polysaccharide is cleaved by an addition of a water molecule. In contrast, lyases cleave the oxygen-aglycone bond in the glycosidic linkages with the loss of a water molecule. The activity of lyases is on the glycosidic linkage adjacent to uronic acid residues in acidic

polysaccharides. During the cleavage, a double bond is introduced to the acid sugar and therefore, the products of lyase activity on EPS are unsaturated oligosaccharide units (Linhardt *et al.*, 1986; Sutherland, 1995)

Fig. 1.8. Mechanisms of lyase and hydrolase activities: (Linhardt *et al.*, 1986; Sutherland, 1995)



The possible functions of *Rhizobium* EPS in the symbiosis may involve intact polysaccharide molecules and/or the products of depolymerase activities. In that sense, an attempt to understand the role of EPS should invariably include a study of depolymerases. In addition, another relevant area worth study is the possible activities of bacterial depolymerases on host plant polymers.

Only a small number of the polysaccharide-producing micro-organisms also yield enzymes degrading the same polymers (Sutherland, 1990). Dunn and Karr (1992) reported that more than 90% of the 34 strains of *Bradyrhizobium japonicum* screened produced depolymerases against an acidic EPS of the corresponding strains. Furthermore, they suggested that the possible role of the microbial enzymes was the removal of surface exopolysaccharides during the infection stage of the nodulation process. However, as evidence for the presence of depolymerases in rhizobia which can act on their own EPS is very rare, these results should be investigated further.

Solheim and Fjellheim (1984) reported the presence of polysaccharide-degrading enzymes in the root extracts of *Trifolium repens* and *Pisum sativum* and the activity of those enzymes on the extracellular polysaccharides produced by their symbionts, *Rhizobium trifolii* and *Rhizobium leguminosarum* respectively. They further noted that the activities on the polysaccharides of

their own symbionts were higher than that on polysaccharides on each other's symbiont.

A rich source of enzymes degrading bacterial exopolysaccharides has proved to be bacteriophages. A number of viral particles contain polysaccharases as part of the particle structure. After the phage infection, the bacterial lysates contain further amounts of the same enzyme in soluble form (Sutherland, 1990).

Higashi and Abe (1978) reported the induction of an EPS depolymerase in *Rhizobium trifolii* by a virulent phage infection. The activity of the enzyme was found to be on EPS containing glucuronic acid. This was proved by Hollingsworth *et al.* (1984) who showed the mode of action of polysaccharide depolymerases from phage lysates of phages 4S and BY15 grown on *Rhizobium trifolii* 4S and 0403 was that of uronic acid lyases (β-elimination).

1.4.2. Bacterial enzymes against plant polymers

In the complex process of the establishment of the symbiotic association between rhizobial bacteria and legume plants and in the subsequent nodule stage, the bacteria interact with a variety of plant polymers. These polymers may be structural polymers like cellulose or storage polymers such as starch. When entering into the plant root, bacterial cells have to pass through plant structures like cell wall which are composed of plant polymers. There is

evidence to prove that, at least in some symbiotic systems, bacterial enzymes play a role of hydrolysing plant structural polymers like cellulose and pectin during the establishment of nodules. However, no report/evidence was hitherto made to show the possible activities of rhizobia against plant storage polymers. In the present work, in addition to the cellulase and pectinase activities, amylase activity of rhizobia is investigated.

1.4.2.1. Rhizobia and plant structural polymers (Cellulase and pectinase activities of rhizobia).

Pectin, a polymer of galacturonic acid, can be found in plant cell walls and in the middle lamella. Cellulose is the major component of plant cell walls and is a homopolymer of D-glucose joined by β-(1,4) linkages. Pectinases (polygalacturonases) and cellulases are the enzymes which break down these polymers. Attempts have been made to investigate the presence/ absence of cellulases and pectinases in rhizobia and the possible roles of these enzymes in symbiosis. Hubbell (1981) and Dazzo and Hubbell (1982) pointed out that if wall degrading enzymes are involved in infection, their production would have to be restricted to account for slow, localized penetration without destruction of the root hair and subsequent abortion of the infection process. This, on the other hand, explains that the presence of cellulases and pectinases in rhizobia, if any, should be only in minute amounts. Hubbel *et al.* (1978) reported low levels of *in vitro* pectolytic enzyme activity of rhizobia. All the limited number of tropical legumes studied by them showed little or

no pectolytic activity, whereas the temparate strains were pectoytic. (They attributed the difference to the observation that temperate legumes are infected through root hairs, whereas the tropical legumes appear to be infected only in the outer cortical cells after the mechanical disruption of surface cell layers.)

Martinez-Molina and Olivares (1982) found indirect evidence for pectolytic enzyme activity in the symbiosis between *Rhizibium meliloti* and *Medicago sativa*. Pectin was added into the rooting medium and subsequently, the number of nodules formed was counted which was found to be less than that in normal conditions. The authors interpreted it as evidence for the presence of bacterial pectinases. It was assumed by the authors that, in normal conditions, bacterial pectinases engage fully in degradation of plant structural pectin and thereby, facilitate a higher nodulation. Martinez-Molina *et al.* (1979) detected *in vitro* cellulase and hemicellulase activities of several symbionts of tropical and temperate legumes. Lopez and Signer (1987) also provided evidence for the production of hemicellulase by *Rhizobium*. Callaham and Torrey (1981) produced strong evidence, obtained from electron micrographs of infection sites of *Trifolium repens* L. by *Rhizobium trifolii*, for the involvement of wall degrading enzymes in infection. They showed a localized degradation of the root hair wall at the site of the infection thread origin and suggested that this was due to the action of rhizobial enzymes.

Electron micrograph observations made by Ridge and Rolfe (1985) on the infection of *Macroptilium atropurpureum* by *Rhizobium* sp.strain ANU240 demonstrated that a region of root hair cell wall is degraded by rhizobia. They suggested that the cell wall is altered by hydrolytic enzymes, either from the host or from the rhizobia. Host root polygalacturonase was suggested as the likely enzyme responsible. Plazinski and Rolfe's (1985) study of *Rhizobium trifolii* strains ANU794, ANU843 and ANU1030 showed pectinase activities of plate cultures.

Baker *et al.* (1989) reported that many cells of *Rhizobium leguminosarum* bv. trifolii attached to the root surface of white clover produce pit erosions in epidermal walls that follow the contour of the bacterium, suggesting that wall-degrading enzymes are associated with the bacterial cell surface itself and/or locally induced in the plant by components of the bacterial surface. Morales *et al.* (1984) reported the induced (though very low) production of cellulase in *Rhizobium trifolii* by a variety of polysaccharides incuding cellulose and hemicellulose. They showed that root extracts of the host plant also can stimulate the production of cellulase. Saleh-Rastin *et al.* (1991) also showed cellulase activities of rhizobia.

Mateos *et al.* (1992) provided a study on the production and several other aspects of pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* bv. trifolii. Using methods sensitive enough to detect the presence of minute quantities of enzymes, they showed the production of three carboxymethyl

cellulase isozymes and one polygalacturonase by the bacterium. These activities were cell bound, with at least some of the activity associated with the cell envelope. The activities were not changed by growth in the presence of the substrates, i.e., carboxymethyl cellulose and polypectate. Furthermore, they found that the symbiotic plasmid, pSym, of the bacterium contributes to its polygalacturonase activity but not to the carboxymethyl cellulase activity. This was consistent with previous results of Morales *et al.* (1984) in which the curing of pSym did not affect cellulose production in *Rhizobium leguminosarum* bv. trifolii 521.

Chalifour and Benhamou (1989) showed the presence of cellulosic β-(1,4)-glucans in root cell walls of Pea (*Pisum sativum* L.) nodules at different stages of infection by *Rhizobium leguminosarum* bv. viceae, as indirect evidence for the cellulase production by the bacterial symbiont. They concluded that a hydrolytic enzyme is involved in the sequence of events from infection thread formation through rhizobial release in the host cell cytoplasm, and that the hydrolytic enzyme is of rhizobial origin.

1.4.2.2. Rhizobia and plant storage polymers (Amylase activity of rhizobia)

Starch is a mixture of linear and branched homopolymers of D-glucose. The glucose molecules of starch are joined together by α-glucosidic linkages and, thus, starch is an α-glucan. The related α-glucan of animal origin is glycogen.

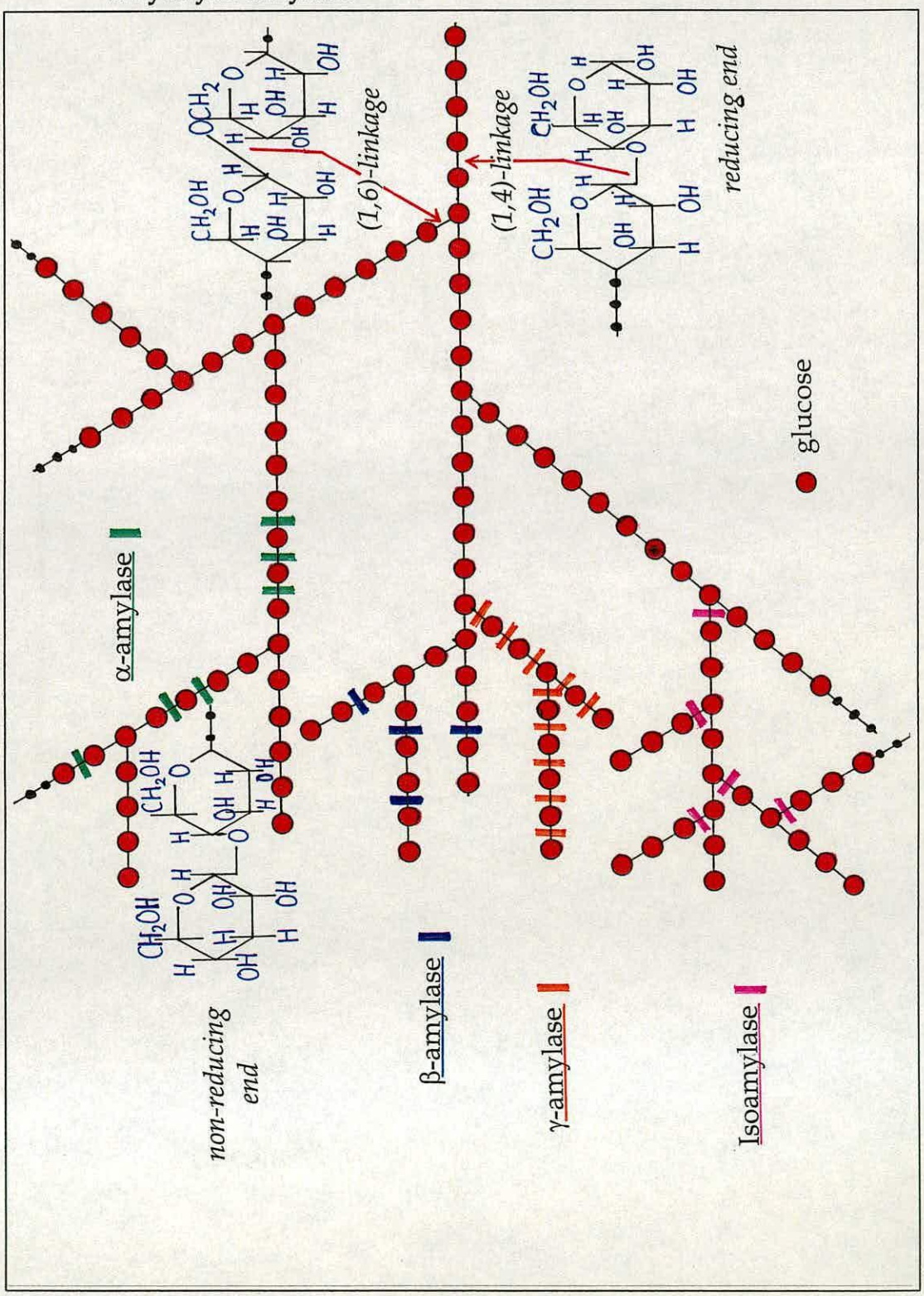
Starch is the main carbohydrate nutritional reserve in higher plants and in certain algae. The linear component (20-25%, depending on source) of starch, amylose, consists of chains of α-(1,4)-D-glucopyranose ranging in degree of polymerization from about $10^{2-4} \times 10^5$. Amylose is water soluble and forms blue-black complexes with iodine. Amylopectin has shorter chains (17-23 units long) of α-(1,4)-D-glucopyranose joined together by α-(1,6) linkages to form a branched structure. It has one α-(1,6) linkage per c. 30 α-(1,4) linkages. The degree of polymerization of amylopectin is between about 10^4 and 4×10^7. Amylopectin is water-insoluble and forms violet or red brown complexes with iodine.

The enzymes which can hydrolyse the α-glucosidic linkages of starch are known as amylolytic enzymes, or α-glucanases. These enzymes occur widely in plants, animals and microbes. There is a wide variation in the properties of different α-glucanases and organisms usually have several enzymes involved in starch hydrolysis (reviewed by Vihinen and Mantsala, 1989)

According to the action on starch, the amylolytic enzymes can be divided into two main categories: endoamylases and exoamylases. Endoamylases hydrolyse the α-glucosidic linkages in the interior of starch molecules while the exo- type of enzymes are responsible for the cleavage of those linkages in the non-reducing end of the polymer chains. Another category can be identified among the amylolytic enzymes when the particular bond which is

hydrolyzed is considered. The enzymes which hydrolyse the α-(1,6) linkages are called debranching enzymes.

Fig. 1.9. Schematic structure of starch and the action pattern of some amylolytic enzymes



There are several groups of amylolytic enzymes in nature and each hydrolyses starch in different ways. Vihinen and Mantsala (1989) pointed out that the properties of amylolytic enzymes vary and the degradation of starch usually requires cooperation of several enzymes because few enzymatic activities can cause complete degradation. Thus, starch-degrading micro-organisms usually possess several amylolytic activities.

α-Amylases (α-(1,4)-D-glucan 4-glucanhydrolase) are the most widely studied amylolytic group of enzymes. These enzymes hydrolyse the internal α-(1,4) linkages of glucan polymer chains. They have little action on terminal α-(1,4) bonds or α-(1,4) bonds adjacent to α-(1,6) branch points. A very small number of α-amylases can also degrade some α-(1,6) bonds (Sakano *et al.*, 1985), but the rates of the reactions are very low. The α-amylases form glucose, maltose and branched dextrins (α-limit dextrins) from amylopectin (and glycogen). They act on amylose to form first poly- and oligosaccharide chains of varying lengths, and then slowly on the intermediate products to form maltose and glucose. α-Amylases are divided into two loose categories according to the degree of hydrolysis of starch (Fukumoto and Okada, 1963). The α-amylases which can hydrolyse 50-60% of starch are called saccharifying α-amylases and those which can hydrolyse about 30-60% are liquefying α-amylases. α-Amylases occur widely among microorganisms grown under a range of different environments including halophiles, alkalophiles, acidophiles and thermophiles. Most of the α-amylases are

extracellular. It is not known why some strains produce intracellular (cytoplasmic) α-amylases because starch cannot penetrate into cells (Vihinen and Mantsala, 1989). Many microbes also produce membrane-bound α-amylases. The same microbe can produce all of these different α-amylases. *Bacillus* species, including *Bacillus subtilis* are well known α-amylase producers.

Vihinen and Mantsala (1989) summarised the physicochemical properties of α-amylases. The pH optimum of α-amylases varies from 2.5 to 10.5, indicative of evolutionary adaptability to different environments. The temperature optima for α-amylases and for the growth of the corresponding organisms are related. The lowest temperature optimum is reported to be 25-30°C for *Fusarium oxysporum* amylase (Chary and Reddy, 1985) and the highest, at least 100°C, for *Bacillus licheniformis* enzyme (Piggott, *et al.*, 1984). Molecular weights of α-amylases vary from 10,000-139,000 and usually for microbial α-amylases are 50,000-60,000. Most metal cations, especially heavy metal ions, sulphydryl group reagents, EDTA [Ethylene diamine tetra acetic acid] and EGTA [Ethylene Glycol-bis(β-aminoethyl Ether)] inhibit α-amylases. As shown by Vallee *et al.* (1959), α-amylase is a metalloenzyme which contains at least one activating and stabilizing Ca^{2+} ion. The amount of bound calcium varies from 1 to about 10 ions and the role of calcium may be to tighten the binding between the domains of α-amylase molecule (Vihinen and Mantsala, 1989).

α-Amylases are known to be multidomain proteins, i.e., the molecules consist of several folding units. However, each α-amylase is believed to have a catalytic domain which consists of a barrel of 8 parallel β-strands surrounded by 8 α-helices, with an extra helix inserted after the sixth β-strand (MacGregor, 1993). The β-strands and the α-helices alternate along the peptide chain and are linked together by irregular loops. Amino acid residues situated on the loops joining the C-terminal end of each β-strand to the N- terminal end of the following α-helix make up the active site of the enzymes. MacGregor (1993) further pointed out that it is now believed such a (β/α)8-barrel also constitutes the catalytic domain of enzymes active on α-(1,6)-glucosidic bonds, and of enzymes with dual specificity for both α-(1,4) and α-(1,6) bonds.

β-Amylases (α-(1,4)-D-glucan maltohydrolase) are exo-enzymes which cleave alternate α-(1,4) linkages from the non-reducing end of a linear α-(1,4)-D-glucan, starting from the penultimate bond. Amylose is degraded into maltose by β-amylase and the action of the enzyme inverts the anomeric configuration of the liberated maltose to β. The enzyme halts at α-(1,6) branch points of amylopectin (and glycogen) producing maltose and β-limit dextrins. β-Amylases can also cleave α-(1,6) and α-(1,3) linkages at a much lower rate. β-Amylases occur commonly in plants, but reports of their occurrence in microbes are limited (Vinhinen and Mantsala, 1989). β-Amylases are extracellular enzymes, although Srivastava (1984) and

Srivastava *et al.* (1984) reported the presence of intracellular β-amylases in some thermophilic bacilli. However, Vihinen and Mantsala (1989) questioned these findings as the reported β-amylases showed such properties as inctivation by EDTA and activation by calcium ions, characteristics which are typical for α-amylases and not found in any other β-amylase. Molecular weights for β-amylases vary from 31,000-160,000 (Vihinen and Mantsala, 1989). *Bacillus polymyxa* and other *Bacillus* species and some clostridia are typical β-amylase producers.

γ-Amylases (glucoamylase, amyloglucosidase, α-(1,4)-D-glucan gluco hydro lase, or exo α-(1,4)-D-glucosidase) are exoenzymes which cleave α-(1,4) linkages consecutively from the non-reducing sugar end of an α-(1,4)-D-glucan to yield β-D-glucose. γ-Amylases can also cleave α-(1,6) and α-(1,3) linkages at much lower rate. Many glucoamylases can hydrolyze pullulan. Glucoamylases are produced by many fungi, but only by a few bacteria, including *Bacillus stearothermophilus* (De Pinto and Campbell, 1968) and *Clostridium thermohydrosulfuricum* (Norman, 1983). Fungal glucoamylases are extracellular while the enzyme is tightly cell-bound in *Flavobacterium* sp. (Pongsawashi and Yagisawa, 1987). In the bacterium *Clostridium thermohydro-sulfuricum* (Norman, 1983), it is both intracellular and membrane bound. Glycoamylases have pH optima in the acidic range and many have temperature optimum at or near 60°C. Their molecular weights vary from 20,000-306,000 (Vihinen and Mantsala, 1989).

Enzymes which hydrolyse the α-(1,6) branch points of amylopectin, glycogen and/or related polysaccharides are known as debranching enzymes (amylo - (1,6)-glucosidase). Pullulanase cleaves the α-(1,6) bonds of pullulan which is a linear glucan consists of maltotriose units joined by α-(1,6) linkages. This group of enzymes is produced by few microbes including *Enterobacter aerogenes* and *Bacillus cereus*.

Isoamylases (glycogen 6-glucoanhydrolase) are also debranching enzymes. They hydrolyse the α-(1,6) linkages of amylopectin, glycogen, various branched dextrins and oligosaccharides but cannot degrade pullulan (Kainuma *et al.*, 1975) and cannot hydrolyse all α-(1,6) linkages in β-limit dextrins (Yokobayashi *et al.*, 1970). Isoamylases may be extra- or intracellular or membrane-bound. They are present in a few microbial strains only, including *Escherichia coli*, the enzyme of which hydrolyzes amylopectin completely (Jeanningros *et al.*, 1976). The isoamylases are usually thermolabile and their pH optimum lies in the acidic range. The molecular weights are usually higher than that of α- or β- amylases, ranging from 65,000-121,000 (Vihinen and Mantsala, 1989).

α-Glucosidases (maltases, α-D-glucoside glucohydrolases) can hydrolyse both α-(1,4) and α-(1,6) linkages, but usually only on short chain oligosaccharides. These enzymes are widely distributed among microorganisms and they liberate α-D-glucose units starting from the non-

reducing end. α-Glucosidases are usually present with other amylolytic enzymes. Many α-glucosidases are intracellular enzymes, though some strains have both intra- and extracellular forms. They have pH optima near pH 7 or above. Molecular weights of α-glucosidases are between 12,000 and 160,000 (Vihinen and Mantsala, 1989). Isomaltases (oligo-(1,6)-glucosidase, dextrin 6-α-D-glucanohydrolase) are similar to α-glucosidases and degrade only α-(1,6) linkages at the non-reducing end of short chain substrates. The presence of isomaltase has been reported in several *Bacillus* species (Wang and Hartman, 1976; Kelly *et al.*, 1980; Fogarty *et al.*, 1985).

Cyclodextrin glucanotransferases (cyclodextrin glycosyltransferases, α-(1,4)-D-glucan 4-α-D-(α-(1,4)-D-glucano)-transferase (cycling), CGTases) form cyclodextrins from starch, amylose and other related polysaccharides. Rings of 6, 7 and 8 glucose units are called α-, β- and γ-cyclodextrins, respectively. CGTases also catalyze the degradation of cyclodextrins (Vihinen and Mantsala, 1989). *Bacillus macerans* is an example of a microbe producing CGTases and these enzymes are found only in bacterial sources. Although CGTases are normally extracellular, the enzyme of *Bacillus macerans* ATCC 8514 (Laszlo *et al.*, 1980) was obtained by the lysis of the cells. The molecular weights of CGTases are between 67,000-145,000 and the temperature optima lie between 45 and 60°C (Vihinen and Mantsala, 1989). In addition to the CGTases, only a few enzymes can degrade cyclodextrin rings. Most cyclodextrin degrading enzymes are α-amylases.

Starch degradation has not been reported as a characteristic feature of rhizobia. However, Idrissi *et al.* (1996) characterised twelve rhizobial isolates from the root nodules of *Ceratonia siliqua* (Carob tree) and three strains out of the twelve were found to be starch utilisers. There is no direct evidence reported so far, to show that starch or amylolytic anzymes of rhizobial (or plant) origin may play a role in the symbiotic association between rhizobia and legume plants. However, unrelated events in symbiosis which are described below, indicate a few instances where a possible involvement may occur.

A pattern of starch accumulation in the cells of indeterminate type of nodules has been shown by some authors (Vasse *et al.*, 1990; Franssen *et al.*, 1992). Amyloplasts first accumulate in the proximal half (about 15 cell layers) of the central tissue of nodules. Afterwards, this zone progressively decreases in size, and in mature nodules it is reduced to two layers of invaded cells of the distal part of the nodule. Tate *et al.* (1994) studied the developmental aspects of the determinate type nodule formation induced by *Rhizobium leguminosarum* bv. phaseoli on *Phaseolus vulgaris* (bean) roots, and described a pattern of amyloplast accumulations in the nodules. They too recognised two major tissue types of the fully developed bean nodule: the peripheral and the central tissues. They observed starch deposition exclusively in uninvaded cells of the central tissue. In early emergent nodules, small starch granules were present in about 10 layers in the proximal part of the nodule. In mature

nodules, they noted amyloplasts in the uninvaded cells interspersed among the invaded ones and in few layers around the most peripheral invaded cells. Starch granules showed a proximal-distal decreasing gradient and were absent in the nodule region distal to the root. The authors noted that "it is tempting to speculate that the differentiation programme induced by the bacteria causes the appearence of amyloplasts in the uninvaded cells of the early emergent nodule, and, that later on, starch is used up by nitrogen-fixing bacteroids in the invaded cells".

Copeland *et al.* (1995) studied the carbon metabolism in chickpea (*Cicer arietinum* L.) nodules formed in the symbiosis with *Rhizobium* sp. (Cicer) CC1192, where the nodule is known to be an indeterminate one. The content of starch was also monitored during the growth of the nodules. They noted an increase of starch content in the nodules to a maximum between 6 and 7 weeks and then a decline to a low concentration. El-Din (1992) studied the symbiosis between soybean and the mutants of *Bradyrhizobium japonicum* which were defective in dicarboxylate transport. The mutants which were unable to accumulate succinate (but possessed normal levels of malate dehydrogenase, fumarase and hydroxybutyrate dehydrogenase) produced ineffective nodules. In the comparison of the ineffective nodules with the wild type nodules, the non-infected cells of ineffective nodules show more prominent starch granules than in the non-infected cells of effective nodules

where they are few in number and quite small. It is significant that the infected cells in both types did not contain starch granules.

1.5. Aims of the project:

Although the *Rhizobium*-legume symbiosis has been a widely studied phenomenon in nature, most of the associations which have received much attention happened to be host plants and their symbionts from temperate regions. A wide range of tropical legumes, ranging form herbs to trees, therefore, are yet to be studied. As most of the well-studied *Rhizobium*-legume associations have distinct features which make each association unique, it is reasonable to assume that associations from tropical climates may have new features which are worth study. The present work is focused on twelve rhizobial isolates from Sri Lanka, particularly the characteristics of their exopolysaccharides and polysaccharide degrading enzymes and comparison of these with the well-characterised isolates.

2. Materials and methods

2.1. Rhizobial strains

The rhizobial strains especially isolated for this study from legumes grown in Sri Lanka and Scotland are given in table 2.1 with information on their host plants.

Table 2.1. Rhizobial strains isolated in the study

Isolate	Legume
Sri lankan rhizobia and their host legumes	
SL2a, SL2b	*Adenanthera pavonina* (Tree)
SL3	*Psophocarpus tetragnolobus* (Vine)
SL4	*Mimosa pudica* (Herb)
SL5	*Albizzia* sp. (Tree)
SL7a, SL7b, SL7c	*Desmodium trifolium* (Herb)
SL7a, SL9b	*Leucaenia leucocephala* (Tree)
SL10	*Acacia* sp.(Tree)
SL11a	*Vigna sinensis* var.(Vine)
SL12	*Sesbania grandiflora* (Tree)
Strains isolated from horticultural legumes (Scotland)	
Rhi-A	Lupin
Rhi-E	Clover
Rhi-G	Pea

Table 2.2 gives a list of the well-defined rhizobial strains provided by other research groups and used in the present study

Table 2.2. Other rhizobial strains used in the study

Strain	Source
Bradyrhizobium H_1	Professor J Beringer, Bristol
Bradyrhizobium H_2	Professor J Beringer, Bristol
Bradyrhizobium H_3	Professor J Beringer, Bristol
Bradyrhizobium USDA110	Professor J Beringer, Bristol
Bradyrhizobium 11.160.M_7	Professor J Beringer, Bristol
Rhizobium AWU237	Professor W Broughton, Geneva
Rhizobium WGR234	Professor W Broughton, Geneva
Rhizobium 4S	Professor F Dazzo, Michigan

2.2. Media

2.2.1. M79-mannitol agar

Mannitol	10.0g
Yeast extract	1.0g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Agar	15.0g
Distilled H_2O	1.0l

M79-mannitol agar was modified by the addition of the following trace elements (mg/l)

$FeCl_3.6H_2O$	2.5
$MnCl_2$	1.0
H_3BO_3	0.01
$ZnSO_4.7H_2O$	0.01
$CoCl_2.7H_2O$	0.01
$CuSO_4.5H_2O$	0.01
$Na_2MoO_4.2H_2O$	0.01

2.2.2. GMS broth

GMS broth (Zevenhuizen, 1986) was used to culture the bacterial isolates in liquid medium. 1l inoculated medium in 2l flasks was incubated for 2-5d on a mechanical shaker at 30rpm and under 30°C. Yeast extract (0.5g/l) was added to the medium in place of biotin and thiamin (see next page).

Mannitol	5.0g
Yeast extract	0.5g
K_2HPO_4	1.0g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	0.05g
Sodium glutamate	1.0g
Distilled H_2O	1.0l
Micronutrients (mg/l)	
$FeCl_3.6H_2O$	2.5
$MnCl_2$	1.0
H_3BO_3	0.01
$ZnSO_4.7H_2O$	0.01
$CoCl_2.7H_2O$	0.01
$CuSO_4.5H_2O$	0.01
$Na_2MoO_4.2H_2O$	0.01

2.3. Methods

2.3.1. Isolation of rhizobia

The legume roots with nodules were rinsed with tap water to remove soil. Nodules were removed onto a clean petri dish using a sterile scalpel and were surface sterilised for 10min using a 10% solution of sodium hypochlorite. The nodules were then rinsed in six changes of sterile distilled water to remove hypochlorite and were homogenised using a sterile glasshomogeniser. The homogenate was streaked onto modified M79-agar and the plates were incubated at 30°C to isolate pure cultures.

2.3.2. Culture methods

Harvesting the exopolysaccharides

After growth of 2-5d in GMS broth, the cultures were centrifuged at 4°C at 11000rpm for 25min. The supernatant was concentrated using a tangential flow filtration apparatus (Millipore) with a 10000 pore size filter. Exopolysaccharide present in the concentrated solution was precipitated using c 2 vol. acetone. The floating masses of exopolysaccharide were removed, excess acetone pressed out, dissolved in water and were dialysed against running tap water. A few drops of formaldehyde was added into the dialysis sac to prevent any enzyme or bacterial degradation. After dialysis, the EPS solutions were frozen at -20°C and were freeze-dried. For NMR and HPLC studies (p. 105 and p. 108), the exopolysaccharides were purified further by redissolving in distilled water, ultracentrifigation, dialysis against distilled water and subsequent freeze-drying.

Cell lysates

The rhizobial liquid cultures were grown for 2-4d. at 30°C and centrifuged as described earlier (p. 90). The cell pellets were suspended in buffer (pH 7.0, 0.02M Tris/HCl) and were sonicated for 4min using an ultrasonicator (MSE) with ice around the sonicator tube and leaving 1min gaps between each minute to stop temperature increase. The sonicated cells were centrifuged at 11000rpm to remove cell debris and were then subjected to ultracentrifugation at 39000rpm for >3h at 4°C. The supernatants were

dialysed against buffer (pH7.0, 0.02M Tris/HCl) in the cold and were concentrated by dialysing against polyethyleneglycol (6000MW) in the cold. The concentrated cell lysates were stored in the freezer at -20°C.

Harvesting the extracellular amylases

The cells of 3d old cultures in GMS broth with added 1% starch were removed by centrifugation at 4°C at 11000rpm for 25min. The supernatant was first concentrated using a tangential flow filtration apparatus (Millipore) with a 10,000 pore size filter. Exopolysaccharides present in the concentrated solutions were then removed by tangential flow filtration with a 100,000 pore size filter. The filtrates, which contained amylases, were dialysed against pH 7.00 Tris buffer (0.01M) at 4°C, subsequently concentrated by dialysis against polyetylene glycol (600MW) and stored at -20°C.

Preparation of periplasm and cytoplasm
(based on Ames *et al.*, 1984)

The cell pellets obtained by centrifugation of 10l of liquid cultures were dissolved in minimum amount of pH7.0 buffer. 100ml of chloroform was added, mixed and left for 10min at room temperature. An equal vol. of buffer was added and the mixtures were centrifuged. The chloroform layers were discarded and the aquous layer that contained the periplasmic contents were dialysed thoroughly against buffer. The pellets were sonicated and the cytoplasmic contents were obtained by the same procedure used to prepare cell lysates.

2.3.3. Characterisation of the isolates

Calcofluor test

The modified M79-mannitol agar medium was supplemented with 0.2%($^{w}/_{v}$) calcofluor, and the bacterial strains were streaked onto this and incubated at 30°C for 2-5d. The colonies were observed under a UV lamp for fluorescence.

Congo-red test

The bacterial strains were inoculated on modified M79-mannitol agar supplemented with 0.0025%($^{w}/_{v}$) Congo-red. After growth for 2-5d., the colonies were observed for the accumulation of the red coloured dye.

Cellulase activity.

Bacterial strains were inoculated along with and across strips of thin cellulose paper (Cigarette paper) laid on modified M79-mannitol agar. Observations were made after 2-7d. incubation at 30°C for the degradation of paper due to cellulase activity. Cell lysates obtained by ultrasonication were also added to tubes containing pH7.0 Tris buffer with granules of cellulose azure (blue) and during incubation at 30°C, were observed for any release of blue colour. Cell lysates were further added to viscous solutions of carboxymethylcellulose and were incubated at 30°C and observed for loss of viscosity.

Pectin hydrolysis

Modified M79-mannitol agar was enriched with 0.02%($^{w}/_{v}$) pectin (citrus origin, Sigma Chemicals). The bacterial strains were streaked onto the medium and after incubation for 7d at 30°C, were flooded with cetavlon. Clear areas adjacent to the colonies in contrast to the white backgrounds indicated pectin degradation. 0.1%($^{w}/_{v}$) separate solutions of pectin and polygalacturonic acid in pH 7 Tris buffer were added with cell lysates of the rhizobial strains and were incubated at 30°C with added sodium azide. Samples were taken against time and TBA assay and reducing sugar tests were used to detect products of possible breakdown of the substrates.

Starch hydrolysis

0.2%($^{w}/_{v}$) soluble starch was added to modified M79-mannitol agar, inoculated with the rhizobial strains and these were incubated for 2-5d. The plates were then flooded with iodine solution and were observed for clear colourless zones around the colonies.

Tolerance of pH

Tolerance of acidic or alkaline pH was determined by inoculating the strains onto M-79 agar plates and GMS broths at pH 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The growth/ absence of growth was recorded after 5d incubation at 30°C.

Salt tolerance

Tolerance to salts was determined on M-79 agar plates containing 0, 1 2, 3 and 4% NaCl or KCl. Growth was recorded after 5d incubation at 30°C.

Hydrolysis of gelatine

M-79 agar plates containing 2% gelatine were inoculated and were incubated for 10d at 30°C. The plates were flooded with 1% $HgCl_2$. Clear zones around the colonies were indicative of gelatinase activity.

Hydrolysis of urea

The appearance of red colour in GMS broth with 0.012% phenol red and 2% urea (Filter sterilised before added into the sterile GMS broth) indicated that urea was hydrolysed.

Reduction of nitrate

Reduction of nitrate to nitrite or N_2 was determined by the following qualitative method. Test organisms were grown for 5d. in GMS broth medium supplemented with 0.1% KNO_3

Growth in the presence of 8% KNO_3

Rhizobial strains were tested for the ability to grow in GMS broth with 8% KNO_3. The incubation was at 30°C for 5d.

H_2S production

The cultures were stab-inoculated into semisolid agar supplemented with 0.03% sodium thiosulphate and 0.025% ferric ammonium citrate and were incubated for 5-7d at 30oC. Blackening of the medium during growth as a result of formation of iron sulphide was regarded as the positive reaction.

2.3.4. Analytical methods

2.3.4.1. General assay techniques

Glucose oxidase test

A colourimetric assay mixture of glucose oxidase, peroxidase and ADTS (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) was prepared according to the following proportions in pH7 Tris/HCl buffer in the cold. (to give 50µl of 0.5mg/ml glucose oxidase, 10µl of 2mg/ml peroxidase and 150µl of 10mg/ml ADTS in 1ml of buffer)

Glucose oxidase	0.0025g
Peroxidase	0.002g
ADTS	0.15g
20mM Tris/HCl	100ml

Into 1ml of the assay solution, 100µl of the samples were added separately, mixed, incubated at 30°C for 1hr and the absorbance was read at 415nm. A

standard series of 1-10µg of glucose in 100µl distilled water was also assayed simultaneously and the absorbance against glucose concentration was plotted. The glucose concentration in unknown samples were calculated using this standard curve.100µl distilled H_2O with no added glucose was used as the reagent blank.

Reducing sugar test (Park and Johnson, 1949)

Reagents

1. Na_2CO_3 in 0.065% KCN
2. Potassium ferricyanide
3. Ferric ammonium sulphate in 0.0.025M H_2SO_4 which contains 0.1%($^{w}/_{v}$) Triton(100)

200µl of the reagent 1 were added to 200µl of samples in tubes of the same diameter and was followed by 200µl of reagent 2. The mixtures were boiled for 15min, cooled in ice water and 1ml of reagent 3 were added. The tubes were left at room temperature for 15min and the absorbances were read at 690nm. A standard series of glucose (1-10µg/ 200µl) was also assayed and the absorbances were plotted against glucose concentration. 200µl distilled water with added reagents was used as the reagent blank. The glucose concentration in the unknown samples were established using the standard curve.

Thiobarbituric acid (TBA) test (Weissbach and Hurwitz, 1959)

TBA test was used for lyase determination.

Reagents

1. 0.025M Periodic acid in 0.125M H_2SO_4
2. 2%($^w/_v$) Sodium arsenite in 0.5M HCl
3. 0.3%($^w/_v$) Thiobarbituric acid (pH= 2.85)

125µl of reagent-1 was added to 100µl samples in 5ml glass tubes and left at room temperature for 10min. 250µl of reagent-2 was added, left for 2min, and 1ml of reagent-3 was added. The tubes were boiled for 10 min and then kept at about 50oC to prevent precipitation prior to the colourimetric reading at 549nm against controls lacking any enzyme.

Protein estimation

A standard series of protein (albumin -bovine, Sigma Chemicals) concentrations ranging from 2µg to 18µg protein in 1ml of distilled water was prepared and stored at 20°C. 1ml unknown protein solutions, in appropriate dilutions, were dispensed into Eppendorfs. 250µl of Bio-Rad reagent (Comassie brilliant blue in phosphoric acid) were added into both the unknowns and standards using Gilson-Microman pipettes and were mixed by gentle inversion avoiding bubbles. The mixtures were then emptied into cuvettes to measure the absorbance at 595nm.

2.3.4.2. Analysis of exopolysaccharides

O-acetyl determination (Hestrin, 1949)

Reagents

1. 2M hydroxylamine monohydrochloride (stored in cold)
2. 3.5M sodium hydroxide
3. 50% v/v conc. HCL in water
4. 0.37M ferric chloride in 0.1M HCl
5. Standard solution (0.04M acetylcholine chloride, pH 4.5, in 0.01M sodium acetate) (stored at 4°C, less than 2 weeks)

1ml of hydroxylamine was mixed with 1ml of alkali in a test tube. 1ml of 0.2%($^{w}/_{v}$) EPS was added, mixed and kept at room temperature for at least 1min. 1ml of acid was added and after mixing, 1ml of iron solution was added. The density of the resultant purple brown colour was promptly determined at 540nm.

The same procedure was carried out for 1ml of the standard solution, and, in measuring the colour intensity, a dilution series of the dark purple brown coloured product was prepared using 0.074M ferric chloride.

Pyruvate determination (Sloneker and Orentas, 1962)

Reagents

1. 0.5% 2, 4-dinitrophenylhydrazine in 2M HCl
2. Ethyl acetate
3. 10%($^{w}/_{v}$) aqueous sodium carbonate.
4. Standard sodium pyruvate in 1M HCl (2-40µg/200µl)

0.2%($^{w}/_{v}$) exopolysaccharide solutions in 1M HCl were hydrolysed at 100°C for 3h. 1ml of dinitrophenyl reagent was added to 1ml of each of the hydrolysates diluted with 1ml distilled water. After mixing, the tubes were left for 5min at room temperature and 5ml aliquots of ethyl acetate were added. The lower (aqueous) layers were removed and discarded. The pyruvate in the upper layers was extracted with 5ml aliquots of sodium carbonate reagent. The absorbance was measured at 375nm. The same procedure was carried out for a standard series (2-40µg/200µl) of sodium pyruvate in 1M HCl.

Uronic acid determination (Blumenkrantz and Asboe-Hansen, 1973)

Reagents

1. 0.0125M disodium tetraborate ($Na_2B_4O_7{\cdot}10H_2O$- Borax) in conc. H_2SO_4
2. 0.15%($^{w}/_{v}$) of m-hydroxybiphenyl (m-phenylphenol) in 0.5% NaOH
3. Standard solution series of glucuronic acid (0-20µg/200µl)

1.2ml of the reagent 1 was added to 200µl of 0.02%($^{w}/_{v}$) exopolysaccharides in glass test tubes in an ice-water bath. After vortexing, the tubes were boiled for 5min and cooled again in the ice-water bath. 20µl of the reagent 2 was then added, vortexed and the absorbance were read at 520nm.

Neutral sugar analysis

Acid hydrolysis of EPS

Method I

20mg of EPS in 800µl of 0.25M H_2SO_4 in deionised water (UHQ-Elga), was hydrolysed overnight at 100°C in a sealed hard glass ampoule. The hydrolysates were then neutralised with c. 0.5vol. Amberlite MB1 resin (Sigma Chemicals). The neutralised hydrolysates were removed with an additional washing of the resin with UHQ (Elga) water. The solutions were then dried under reduced pressure using a vacuum rotor-evaporator, re-dissolved in about 200µl of UHQ (Elga) water and were minifuged in Eppendorf tubes. The supernatants were stored in the freezer.

Method II

20mg of each EPS separately with 2ml trifluoro-acetic acid (2M) was hydrolysed at 120°C for 3h. The hydrolysates were dried using a vaccum-evaporator, redissolved in 0.5ml of of UHQ (Elga) water, minifuged and the supernatents were kept at -20°C.

HPLC analysis of EPS acid hydrolysates

Method I

After filtration though 0.45μm membrane filters (Millipore), the acid hydrolysates of exopolysaccharides were subjected to High Performance Liquid Chromatographic (HPLC) analysis using a SCX-lead cartridge (30x0.75cm microbore column, Brownlee Laboratories Inc.) at a flow rate of 0.2ml min^{-1} with degassed UHQ (Elga) deionised water. The injected sample sizes were about 10μl. 0.01M D-glucose, D-galactose, D-mannose, L-fucose and L-rhamnose were used as standards.

Method II

A CarboPac PA1 (Dionex) column (250mm x 4mm) was used in conjunction with standard Dionex hardware to analyse the hydrolysates of exopoly-saccharides. The sample size was 20μl and the flow rate was at 1ml min^{-1}. Calibration was performed using the following standard monosaccharides: fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, glucuronic acid and galacturonic acid.

Paper chromatography of EPS acid hydrolysates

The acid hydrolysates of EPS were loaded onto chromatography paper (Whatman, No. 1) and run for about 24-36h along with glucose, galactose, mannose, fucose and rhamnose as standards, with a descending solvent

system of butanol: pyridine: water (6: 4: 3). The chromatograms were developed by the alkaline silver nitrate method described by Trevelyan *et al.* (1950).

^{1}H- NMR (Nuclear Magnetic Resonance) analysis of EPS

5mg of each EPS was dissolved in 1ml of D_2O and used as samples for ^{1}H NMR analysis, at 298°K, by a Varian Inova-600 spectrometer operating at 599.9MHz.

2.3.5. Enzyme assays

Glycosidase activity

0.01M solutions of the following glycoside substrates were prepared.

O-nitrophenyl-α-D-glucopyranoside, *O*NP-β-D-glucopyranoside
*O*NP-α-D-galactopyranoside, *O*NP-β-D-galactopyranoside
*O*NP-α-D-mannopyranoside, *O*NP-β-D-mannopyranoside
*O*NP-α-L-rhamnopyranoside

100µl of nitrophenyl glycosides were added separately to 1.5ml of 20mM pH7 Tris/HCl buffer. After mixing, 25 to 200µl cell lysates -depending upon the protein content of lysate- were added separately. A similar mixture with no enzyme was used as the reagent blank and the absorbance due to release of nitrophenol was recorded against time, at E405nm. Controls with enzymes but no substrate were also prepared. The spectrophotometric readings were

plotted against time, absorbance of the nitrophenol released by a milligram of proteins per minute were calculated and those values were used to estimate the specific activities of the cell lysates against nitrophenyl-glycosides.

Succeptibility of EPS to succinoglycan depolymerase

0.5ml of EPS solution (6mg in 1ml d. H_2O) and 100µl succinoglycan depolymerase (supplied by Professor M Hisamatsu, MIE University, Kamihama, Japan) were incubated for 7d. in 1ml of 0.05M phosphate buffer (pH 6.4) at 30°C. Liberation of reducing sugar due to succinoglycan depolymerase activity was tested by the Park and Johnson (1949) method.

Amylase activity

100µl samples were added separately to 2ml of 0.2%($^{w}/_{v}$) starch solutions (in pH7 Tris/HCl buffer) into which 40µl of iodine (Gram's iodine stock solution) was previously added to make it blue. Loss of colour which was indicative of amylolytic activity was read against time at 650nm. A similar preparation with no enzyme added was used as the control which gave the maximum colour intensity and each assay reading against time was deducted from the maximum reading and plotted against time. Amylase specific activity was taken as the loss of colour units per min per 1mg of protein.

100µl and 200µl samples were taken from starch hydrolysis assay mixtures against time and were used in the glucose oxidase test and reducing sugar test respectively. Glucose or total reducing sugar released against time was plotted. The release of glucose and the release of total reducing sugars per min per 1mg of protein were taken as the amylase specific activities.

2.3.6. Analysis of starch hydrolysates

Cell lysates and a few drops of sodium azide were mixed with 0.2%($^{w}/_{v}$) starch in 20mM pH 7 Tris/HCl buffer in dialysis sacs. The sacs were submerged in about 10 volumes of distilled water with azide and were shaken for 12h at 30°C. The outer solutions, into which the products of starch hydrolysis migrated through the dialysis sacs, were concentrated by rotary evaporation under reduced pressure and then, were re-dissolved in about 200µl of deionised UHQ (Elga) water. Paper chromatograms were obtained by running the samples alongside with glucose, maltose and maltotriose as standards and butanol: pyridine: water (6: 4: 3) as the solvent. The silver nitrate method (Trevelyan *et al.*, 1950) was used to develop the chromatograms.

HPLC studies were made using the Gilson system with a TSK G1000PW column and refractive index monitoring. Prior to runs in the HPLC system, the starch hydrolysate samples were filtered through 0.45µm filters

(Milipore). Glucose, maltose and maltotriose were used as standards. The starch hydrolysis products were also analysed by gel filtration chromatography on Biogel-P2 column. Maltose, maltotriose and maltohexose were used as standards.

2.3.7. Protein purification

Salting-out (Ammonium sulphate precipitation)

Into 25ml samples of crude cell lysates kept in the cold, the amounts of ammonium sulphate (BDH- enzyme work grade) needed to give 10, 20, 30, 40, 50, 60, 70 and 80%($^{w}/_{v}$) strength at 0-4°C were added separately. While ammonium sulphate was gradually added, the solutions were stirred continuously and the stirring was continued for another 30min. The solutions were centrifuged at 4°C at 36500g for 25min and after the supernatants were decanted and drained off, the precipitates were dissolved in pH7.0, 0.02M Tris/HCl buffer and dialysed against several changes of the same buffer in the cold until all the salt was removed. The enzyme activities of each fraction were estimated and the range of ammonium sulphate concentrations which precipitated the target protein was established. This was used to salt-out the protein from the bulk stocks of cell lysates.

Ion-exchange chromatography

Ion exchange chromatography was employed in the purification of amylases using an anion exchange column of pre-swollen microgranular

diethylaminoethyl (DEAE) cellulose (Whatman). The protein solutions (usually obtained by ammonium sulphate step) were applied to the column ($3cm^2$ x 50cm) which was eluted with pH 7.0, 0.02M Tris buffer. The retained proteins were then eluted as fractions using a gradient increase of NaCl in buffer. Protein concentrations in alternate 1ml fractions were assessed by spectrophotometry using a quartz cuvette at 280nm.

Affinity chromatography

Glucose-agarose and maltose-agarose (Sigma Chemicals) columns were used in the purification of amylases. After the application of protein solutions obtained from ion exchange chromatography onto the affinity columns ($1.5cm^2$ x 10cm), the columns were washed with buffer and the adsorbed proteins were eluted using 1M NaCl in buffer.

Gel-electrophoresis (Laemmli,1970)

Resolving gel

Polyacrylamide stock solution	10.0ml
pH8.8, 1.5M Tris buffer with SDS	7.5ml
Distilled water	12.5ml

The mixture was degassed briefly and the following solutions were added.

N',N',N',N'-Tetramethylethylenediamine (TEMED)	45µl
10% Ammonium persulphate	200µl

Stalking gel

Polyacrylamide stock solution	4.5ml
pH6.8, 0.5M Tris buffer with SDS	7.5ml
Distilled water	18.0ml

The mixture was degassed briefly and the followings were added.

N',N',N',N'-Tetramethylethylenediamine (TEMED)	50µl
10% Ammonium persulphate	150µl

Sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% polyacrylamide resolving gel and 4.5% polyacrylamide stalking gell. After pouring the resolving gel into the glass cassette, to get rid of bubbles, a layer of butan-2-ol (saturated with dist. H_2O) was added onto the top of the resolving gel, to be poured off after the gell had settled. The top of the resolving gel was then washed with distilled water and with little stalking gel solution. The stalking gell was poured in and the comb was placed in for making wells. The running buffer (pH 9.5) of the following composition (g/l) was prepared and the settled gel was ran under 100mV constant voltage for about 1hr before the loading of proteins.

Tris base	3.0g
Glycine	14.4g
Sodium dodecyl sulphate	1.0g

Volumes of each sample which contains about approximately 10μg of protein was mixed with an equal volume of Laemmli sample buffer (0.25M, pH 6.8 Tris/HCl with 2% SDS, 10% glycerol, 0.002% Bromophenol blue and 0.2M β-Mercaptoethanol) and were boiled for 5min. A mixture of molecular weight marker proteins were also prepared in the same manner. After minifuging to remove any debris, the samples were loaded into the wells and the standard was loaded alongside. First the gel was run at 10mA (constant current) until the proteins reach the resolving gell and from then on until the blue dye front reach about 1cm from the bottom of the gel, at 20mA. Then the gel was removed carefully, the stalking portion was discarded and was stained by the silver nitrate method using the following reagents.

Fixative 1 (40% methyl alcohol, 10% acetic acid in distilled H2O)
Fixative 1 (10% methyl alcohol, 5% acetic acid in distilled H_2O)
Oxidant (0.2g $K_2Cr_2O_7$, 40μl HNO_3 in 200ml distilled H_2O)
Silver reagent (0.4g silver nitrate in 200ml distilled H_2O)
Developer (30g Na2CO3, 0.5ml formaldehyde in 1l distilled water)
Stopper (0.5% acetic acid)

The gels which were between 0.5-1.0mm thick were soaked in 400ml Fixative 1 on a slow shaker. After 30min, they were transferred into 400ml Fixative 2 for 10min followed by another 400ml for 10 more min. Then, the gels were transferred into the oxidant for 5min after three successive 5min soaking in

distilled water. From the oxidant they were put in the silver reagent for 20min and afterwards, 1min in distilled water. The developer was used between 1-5min until the bands were visible properly and further staining was stopped by using the stopper solution. The stained gel was mounted on thick filter paper soaked with water and was dried using a hot-vacuum gel-drier.

3. Results and Discussion

3.1. Isolation and characterisation of rhizobia from root nodules

Jordan (1984) described the bacteria which belong to the Gram-negative bacterial family Rhizobiaceae as rod-shaped motile cells with one polar or sub-polar flagellum or 2-6 peritrichous flagella. They are aerobic and produce extracellular polysaccharides during growth on carbohydrate-containing media. Identification of rhizobia is difficult when compared to most other bacterial groups. Elkan and Bunn (1992) pointed out that rhizobia are not easily identified when isolated from soil, but can be isolated from nodules and confirmation of isolates should be made by proper plant inoculation tests. The mol% of G+C of DNA is the main and reliable characteristic in identification.

Nodule rhizobia of each of the Sri Lankan legumes under study were isolated from a collection of nodules from each of a number of individual plants of the same species. Therefore, there was a possibility of the presence of more than one rhizobial strain per legume. Accordingly, nodules of some of the legumes gave several rhizobial strains which were selectively isolated on rhizobial specific medium. The table 3.1 shows the isolates of different Sri Lankan legumes.

Table 3.1. Rhizobial isolates from Sri Lankan legumes

Legume	Isolate
Adenanthera pavonina	SL2a, SL2b
Psophocarpus tetragnolobus	SL3
Mimosa pudica	SL4
Albizzia sp.	SL5
Desmodium trifolium	SL7a, SL7b, SL7c
Leucaenia leucocephala	SL9a, SL9b
Acacia sp.	SL10
Vigna sinensis var.	SL11a
Sesbania grandiflora	SL12
Lupin sp.	Rhi-A
Clover sp.	Rhi-E
Pea sp.	Rhi-G

All the bacterial isolates of the present study were Gram negative and motile. All but two strains grew well on GMS agar producing exopolysaccharides extensively. SL7c and SL11a showed a lesser growth. Table 3.2 shows some characteristics of the new isolates along with those of well defined strains. All the strains were catalase-positive while 85% were positive for oxidase test. There was a variability in urease reaction. About half the isolates were capable of hydrolysing urea and a similar percentage were able to utilise citrate. Neither the new isolates nor the defined strains were able to hydrolyse gelatine. 36% of the strains were capable of producing H_2S from Kligler slants. There was variability in the ability to reduce nitrate; only 40% of the strains were unable to reduce nitrate. The rest reduced nitrate to different levels. 28% of the strains could reduce nitrate to nitrite while a few

among them showed only a weak reaction. 20% of the strains showed the ability to reduce nitrate completely to nitrogen gas. The anaerobic condition in the bottom of culture tubes facilitated the nitrate reduction reaction.

Table 3.2. Some biochemical characteristics of the rhizobial strains

Strains	Catalase	Oxidase	Urease	Citrate	Gelatinase	H_2S	NO_3 reduction
SL2a	+	+	-	-	-	-	+
SL2b	+	+	-	-	-	+	-
SL3	+	+	+	+	-	+	-
SL4	+	+	+	+	-	-	+
SL5	+	+	-	-	-	+	-
SL7a	+	+	+	-	-	-	-
SL7b	+	-	-	+	-	-	-
SL7c	+	+	-	+	-	+	w+
SL9a	+	-	-	-	-	-	+
SL9b	+	+	+	+	-	+	+d
SL10	+	-	+	+	-	-	-
SL11a	+	+		-	-	-	+d
SL12	+	+	+	+	-	+	-
Rhi-A	+	+	+	-	-	-	+
Rhi-C	+	+	-	-	-	-	+
Rhi-E	+	+	+	-	-	+	+d
Rhi-G	+	+	+	-	-	+	w+
H1	+	+	-	+	-	-	w+
H2	+	+	-	-	-	-	-
H3	+	+	+	-	-	-	+
USDA110	+	+	+	-	-	-	+d
11.160.M_7	+	-	-	-	-	+	+
WGR234	+	+	+	+	-	-	+d
AWU237	+	+	-	+	-	-	-
4S	+	+	+	+	-	-	-

+d= *denitrification (complete reduction of nitrate)*, w+=*weakly positive*

Table 3.3. Salt tolerance

	NaCl				KCl			
	1%	2%	3%	4%	1%	2%	3%	4%
SL2a	+	+	+	-	+	+	+	-
SL2b	+	-	-	-	+	-	-	-
SL3	+	-	-	-	-	-	-	-
SL4	+	+	+	-	+	+	+	-
SL5	+	+	+	-	+	+	+	-
SL7a	+	+	+	-	+	+	+	-
SL7b	+	+	+	-	+	+	+	-
SL7c	+	+	+	+	+	+	+	+
SL9a	+	+	+	+	+	+	+	+
SL9b	+	+	+	-	+	+	+	-
SL10	+	+	+	-	+	+	+	-
SL11a	+	+	+	-	+	+	+	+
SL12	+	+	+	-	+	+	+	-
Rhi-A	+	-	-	-	-	-	-	-
Rhi-C	+	+	-	-	+	+	-	-
Rhi-E	+	+	+	-	+	+	+	-
Rhi-G	+	+	+	-	+	+	+	-
H_1	+	+	+	-	+	+	+	-
H_2	+	+	+	-	+	+	+	-
H_3	+	+	+	-	+	+	+	-
USDA110	+	+	+	-	+	+	+	-
11-160-M_7	+	+	+	-	+	+	+	-
WGR234	+	+	+	-	+	+	+	-
AWU237	+	+	+	+	+	+	+	+
4S	+	-	-	-	+	-	-	-

The denitrification activity upon nitrate in intact nodules of *Medicago sativa* L. cv. Aragon nodulated by *Rhizobium meliloti* was studied by Garcia-Plazaola *et al.* (1995). They reported a very low activity which was only 0.05% of the nitrogen-fixation rate of the nodules and which, therefore, had a very limited

impact in normal circumstances. The denitrification activity of the nodule bacteroids was only 2% of the potential activity of isolated bacterium. However, the physiological importance of denitrification in nodules remains to be understood (Garcia-Plazaola *et al.*, 1995).

Table 3.4. pH tolerance

Strains	pH 4.6		pH 5.0		pH 6.0		pH 7.0		pH 8.0		pH 9.0		pH 10.0	
	s	l	s	l	s	l	s	l	s	l	s	l	s	l
SL2a	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL2b	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL3	-	-	-	-	+	+	+	+	+	+	+	+	-	-
SL4	-	-	-	-	+	+	+	+	+	+	-	-	-	-
SL5	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL7a	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL7b	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL7c	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL9a	-	-	-	-	+	+	+	+	+	+	-	-	-	-
SL9b	-	-	-	-	+	+	+	+	+	+	+	+	-	-
SL10	-	-	-	-	+	+	+	+	-	-	-	-	-	-
SL11a	-	-	-	-	+	+	+	+	+	+	-	-	-	-
SL12	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Rhi-A	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Rhi-C	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Rhi-E	-	-	-	-	+	+	+	+	+	+	-	-	-	-
RhiG	-	-	-	-	+	+	+	+	-	-	-	-	-	-
H_1	-	-	-	-	+	+	+	+	+	+	-	-	-	-
H_2	-	-	-	-	+	+	+	+	+	+	-	-	-	-
H_3	-	-	-	-	+	+	+	+	-	-	-	-	-	-
USDA110	-	-	-	-	+	+	+	+	-	-	-	-	-	-
11-160-M_7	-	-	-	-	+	+	+	+	-	-	-	-	-	-
WGR234	-	-	-	-	+	+	+	+	+	+	+	+	+	+
AWU237	-	-	-	-	+	+	+	+	-	-	-	-	-	-
4S	-	-	-	-	+	+	+	+	-	-	-	-	-	-

s = *solid culture*, l = *liquid culture*

The strains were tested for the ability to grow on media with added NaCl or KCl. The vast majority of the strains showed a consistent pattern by being able to grow on media up to 3% NaCl or KCl concentrations (Table 3.3). Only a few strains could grow on 4% salt concentration. Strains SL2b, SL3 Rhi-A and 4S failed to grow on salt concentrations above 1% while the strains SL7c, SL9a, SL11a and AWU237 could grow on media with 4% salt. None of the strains could tolerate 8% KNO_3 concentration in the medium.

There was not much variability in the pH tolerance of the isolates of the present study. The optimal pH for all of them was between pH 6.0 and 7.0 (Table 3.4). None of the isolates could grow in acidic pH levels below 6.0. About 45% of the strains could grow at pH values above 7.0. SL3 and SL9b could grow at pH 9 while WGR234 showed the ability to grow at pH 10.0.

Smit *et al.* (1987) and Mateos *et al.* (1994) showed that extracellular cellulose microfibrils of rhizobia help to form a firm attachment of bacterial cells onto host root hair surface. Rhizobia which produce cellulose microfibrils can acquire fluorescence from media containing calcofluor white. Cellulose microfibril-producing colonies are also able to absorb Congo-red from media (Zevenhuizen *et al.*, 1986). The bacterial strains of the present study were tested separately on Calcofluor and Congo-red containing media. They neither showed the ability to absorb Congo-red nor had the ability to acquire fluorescence from Calcofluor indicating the absence of cellulose microfibrils.

The importance of cellulose microfibrils in the attachment of rhizobia onto the host root surface had been demonstrated mostly on *Rhizobium leguminosarum* and that too was of a secondary importance, i.e., to make the attachment firm. Vesper *et al.* (1987) and Loh *et al.* (1993) showed that the mutants lacking cellulose microfibrils show little or no reduction in the ability to nodulate. The present study also confirmed that the lack of cellulose microfibrils was not determinant in the ability to develop nodulation.

The basic characteristics of the Sri Lankan rhizobial isolates and strains isolated from horticultural legumes in Scotland did not show a significant difference from the well-characterised rhizobial strains used in the study. Idrissi *et al.* (1996) reported a characterisation study of 12 rhizobial strains isolated from root nodules of *Ceratonia siliqua.* Almost all the characteristics of the rhizobia of the present study were similar to those studied by Idrissi *et al.* (1996). The only exception was the inability of the isolates of the present study to hydrolyse gelatine. 60% of the strains studied by Idrissi *et al.* (1996) were gelatinase positive.

In the various stages of nodule development and during the subsequent nodule life, the symbiotic rhizobia have to interact physically with different host plant compounds like cellulose and pectin. However, cellulase and pectinase/ polygalacturonase activities were not reported as widespread and

prominant characteristics of Rhizobiaceae. Previous studies indicated that the presence of these enzymes, if any, was limited to very low amounts (Hubbell *et al.*, 1978; Hubbell, 1981; Dazzo and Hubbell, 1982; Morales *et al.*, 1984). Low cellulase or pectinase activities help rhizobia for a successful entry into the host tissues while high concentrations would not help in nodulation as it would cause extensive damage to host tissues. Although several methods were used to detect cellulase or pectinase/ polygalacturonase activities, the rhizobial strains used in the present study failed to show the presence of any of these enzymes.

3.2. Glycosidase activities and possible carbon sources

It has been shown that the nodules formed by *Rhizobium* mutants incapable of C_4-dicarboxylic acid transport are ineffective (Ronson *et al.*,1981; Finan *et al.*, 1983; Boulton *et al.*, 1986; Day and Copland, 1991). Conversion of nitrogen into ammonia is an energy-intensive enzymatic reaction and C_4-dicarboxylic acids have been generally considered to be the main energy bearing compounds supplied by the plant to the bacteroids in the root nodule facilitating effective nitrogen-fixation. However, little is known about the nature of the nutrients utilised by rhizobia prior to the nodule life, i.e. in the free living stage and during root invasion, colonisation and nodule formation. Jimenez-Zurdo *et al.* (1997) showed that *Rhizobium meliloti* proline dehydrogenase gene (*putA*) is expressed during the different steps of the symbiotic association with alfafa, but, not in differentiated bacteroids. They

suggested that proline and or its metabolic precursors are an important energy sources during the establishment of the symbiotic association, but not in supporting the actual nitrogen-fixation.

Tests done in various laboratories, mostly for identification purposes, have revealed that rhizobia can utilise a wide range of carbon sources. Utilization of nitrate is also a common activity of many rhizobia. It has been also found that most of these activities are plasmid encoded (Finan *et al.*, 1986; Watson *et al.*, 1988; Charles *et al.*, 1990; Charles and Finan, 1991). However, little is known about the occurrence of these activities during the process of nodule development.

Glycoside linkages are common to a wide range of substances found in nature. A large number of plant storage and structural compounds as well as bacterial compounds have glycoside linkages. It is known that plant pathogens possess glycoside hydrolases which hydrolyse glycoside linkages in plant structural and storage carbohydrates. Abe and Higashi (1982) reported the presence of β-glucosidase and β-galactosidase in *Rhizobium trifolii* 4S. β-Glucosidase from this bacterium exhibited strong hydrolytic activity on cellobiose, sophorose and laminariobiose while β-galactosidase degraded lactose and galactoside-arabinoside. Both enzymes showed little activity on polysaccharides.

In the present work, it was attempted to establish whether the rhizobial strains possess glycoside hydrolase activities. Nitrophenyl glycosides are a group of compounds widely used in diagnostic bacteriology. Each nitrophenyl glycoside molecule has a glycoside bond which can be hydrolysed to release nitrophenol. In the nitrophenyl glycoside test, glycosidase activity is estimated using the spectrophotometer absorbance of the nitrophenol at 405nm.

Crude cell lysates of the rhizobial strains were tested against *O*-nitrophenyl-α-D-glucopyranoside, *O*NP-β-D-glucopyranoside, *O*NP-α-D-galactopyrano side, *O*NP -β-D-galactopyranoside, *O*NP-α-D-mannopyranoside, *O*NP-β-D-mannopyranoside, *O*NP-α-D-maltopyranoside and *O*NP-α-L-rhamnopyrano side, at 30°C and pH 7.00. Positive lysates started giving the colour reaction mostly within 10-15min. The absorbance at 405nm was read against time, plotted and the increase of absorbance min^{-1}. was derived from the curves. Figure 3.1 shows the curves obtained from the β-glucosidase assay. The following equation was used to calculate the glycosidase specific activities.

$C = (E405nm/ \varepsilon) L$

where,

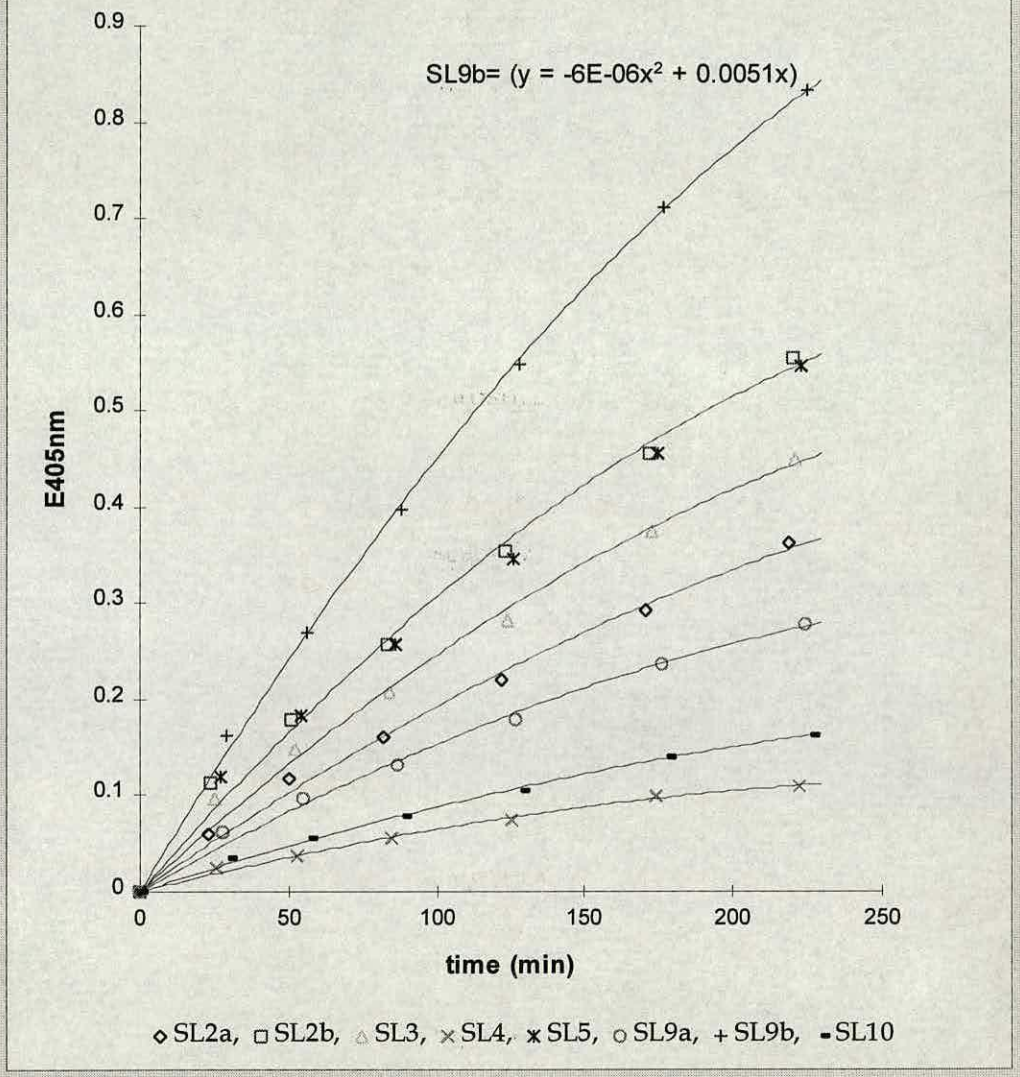
C = concentration of nitrophenol released into the assay mixture (mol ml^{-1})

E405nm = absorbance at 405nm

ε = molar extinction coefficient of nitrophenol = 18.5cm^2 μmol^{-1}

L = light path in the spectrophotometer cuvette = 1cm

Figure 3.1. β-glucosidase activity: absorbance *vs.* time



The equation which was used to calculate the increase of absorbance min⁻¹ is shown with SL9b-curve as an example, where y= increase of absorbance, x= time change (min)

The amount (mol) of nitrophenol released was calculated using the C value and the total volume of the assay mixture. As the protein concentrations of the cell lysates were known, it was possible to calculate μmol nitrophenol released mg^{-1} of cell lysate protein. Glycosidase specific activity of each rhizobial strain was expressed as the number of μmol. of the relevant

nitrophenyl glycoside hydrolysed $min^{-1}.mg^{-1}$.cell lysate protein, at 30°C and pH 7.0. Table 3.5a shows the specific activities of the Sri Lankan strains.

All the Sri Lankan strains, except SL7c and SL12, showed some glycosidase activity. Among the eight different NP-glycosides tested, only three, namely, *O*-nitrophenyl-α-D-mannopyranoside, *O*-nitrophenyl-β-D-mannopyranoside and *O*-nitrophenyl-α-L-rhamnopyranoside were not hydrolysed by any Sri Lankan rhizobia. SL9b and SL3 showed α-galactosidase and α-maltosidase activities. The majority of the rhizobia showed α-glucosidase, β-glucosidase and β-galactosidase activities. However, there was a large variation in the degree of activity of different glycosidases. In general, β-glucosidase activities of the strains were strongest, followed by α-glucosidase while β-galactosidase activities were relatively weak. Table 3.5b compares the different glycosidase activities among the Sri Lankan rhizobia, taking β-glucosidase activity of SL4 as 1. Abe and Higashi (1982) made a similar comparison with different glycosidase activities of a single rhizobial strain, *Rhizobium trifolii* 4S. They too related the other glycosidase activities to β-glucosidase activity which was regarded as 1 and found that all except β-galactosidase activity, which was 1.262, were less than 1. All the Sri Lankan strains followed a similar pattern and the only exception was the low degree of β-galactosidase activity.

Table 3.5a. Nitrophenyl glycosidase specific activities of Sri Lankan rhizobial strains

Strains	a	b	c	d	e	f	g	h
SL2a	0	0	0	0.0005	0	0	0	0
SL2b	0	0.0237	0	0.0011	0	0	0	0
SL3	0.0106	0.0039	0	0.0004	0.002	0	0	0
SL4	0.0016	0.0394	0	0.0003	0	0	0	0
SL5	0	0.0262	0	0.0013	0	0	0	0
SL7b	0.0007	0	0	0	0	0	0	0
SL7c	0	0	0	0	0	0	0	0
SL9a	0	0.0166	0	0.0007	0	0	0	0
SL9b	0.015	0.002	0.0165	0.0012	0	0	0	0
SL10	0.0019	0.0037	0	0.0003	0	0	0	0
SL11a	0.0083	0.0019	0	0	0	0	0	0
SL12	0	0	0	0	0	0	0	0

-Note for tables 3.5a and 3.5b-

Specific activities (μmol $min^{-1}mg^{-1}$ protein)
a = α-Glucosidase (against *O*-nitrophenyl-α-D-glucopyranoside)
b = β-Glucosidase (against *O*-nitrophenyl-β-D-glucopyranoside)
c = α-Galactosidase (against *O*-nitrophenyl-α-D-galactopyranoside)
d = β-Galactosidase (against *O*-nitrophenyl-β-D-galactopyranoside)
e = α-maltosidase (against *O*-nitrophenyl-α-D-maltopyrnoside)
f = α-mannosidase (against *O*-nitrophenyl-α-D-mannopyranoside)
g = β-mannosidase (against *O*-nitrophenyl-β-D-mannopyranoside)
h = α-rhamnosidase (against *O*-nitrophenyl-α-L-rhamnopyranoside)

Table 3.5b. Relative glycosidase activities of Sri Lankan rhizobia when the SL4 β-glucosidase specific activity was taken as 1

	SL2a	SL2b	SL3	SL4	SL5	SL7b	SL7c	SL9a	SL9b	SL10	SL11a	SL12
a			0.27	0.04		0.02			0.38	0.05	0.21	
b		0.6	0.1	1	0.66			0.42	0.05	0.09	0.05	
c									0.42			
d	0.01	0.03	0.01	0.01	0.03			0.02	0.03	0.01		
e			0.05									
f												
g												
h												

Plant cell walls contain pectin. From the experiments carried out prior to the glycoside hydrolysis tests, it was found in the present study that none of the strains were able to hydrolyse pectin or polygalacturonic acid. The rhizobial cultures were grown on pectin-agar media and were flooded with $HgCl_2$-reagent, but no clear zones were observed around the colonies. Cell lysates were incubated with polygalacturonic acid solutions and samples were subsequently used in the reducing sugar test. However, no release of reducing sugar was observed, indicating that the cell lysates do not possess polygalacturonase activity.

Although the strains showed β-glucosidase activities to a significant degree, none were able to breakdown cellulose or carboxymethyl cellulose. The β-glucosidase and β-galactosidase isolated by Abe and Higashi (1982) from *Rhizobium trifolii* 4S, which were found in abundance in the rhizobial strain, were tested by them against some polysaccharides of plant origin but only very low activities were observed. Information about plant structural compounds hydrolysed by rhizobia is very limited and the available literature also describe highly localised, low degree of activities in the initial stages of nodule development. Considering the inability of the rhizobial strains used in the present study to hydrolyse plant polymers, even when they have enzymes like β-glucosidase, it can be concluded that degradation of plant polymers is not a prominent activity. It should be noted that, on the

other hand, such activities by a symbiont within a plant host could be rather harmful.

The question arises about the importance of the presence of these glycosidases to the rhizobia and/or to the symbiotic association. In order to establish the particular α or β linkage those glycosidases are specific for, and to find out whether they utilise oligosaccharides as carbon sources, the cell lysates were tested against maltose, lactose, cellobiose and sophorose.

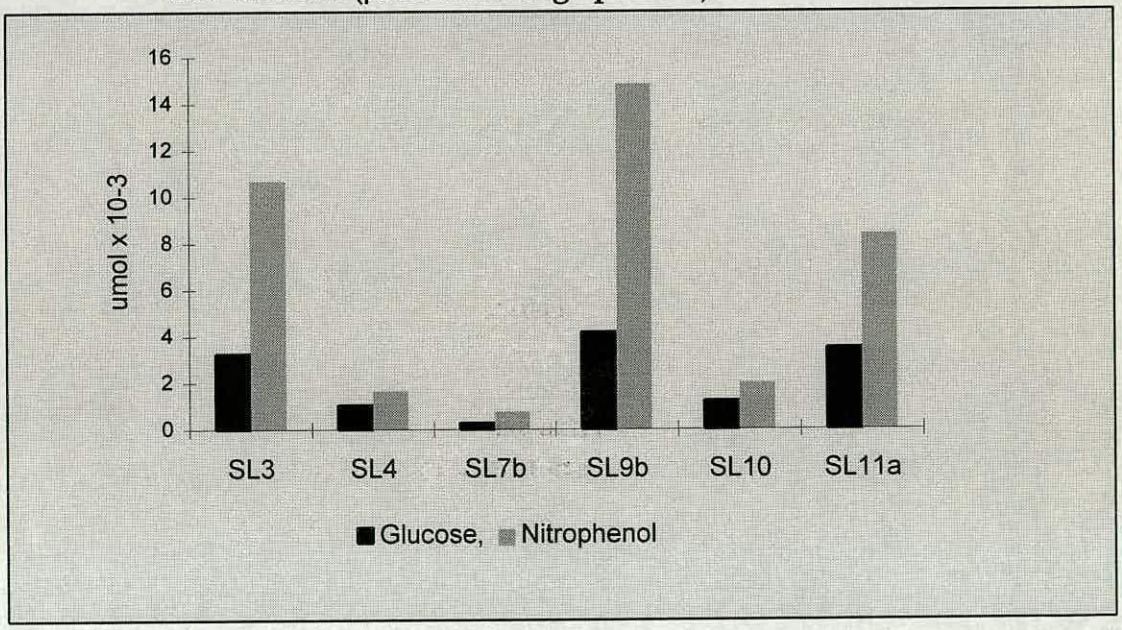
100µl of cell lysates of the rhizobial strains which showed α-glucosidase against *O*-nitrophenyl-α-D-glucopyranoside were incubated separately at 30°C with 0.01M maltose in 0.02M, pH7.0 Tris buffer. Samples (100µl) were taken for the glucose oxidase test. The µmol glucose released min^{-1} mg^{-1} protein in cell lysate was considered as the α-glucosidase specific activity of the particular strain against maltose at the specified temperature and pH. The results are shown in the table 3.6.

Table 3.6. α-glucosidase specific activities against maltose

Strains	Specific activity x 10^{-3} (µmol $min^{-1}mg^{-1}$)
SL3	3.28
SL4	1.07
SL7b	0.28
SL9b	4.19
SL10	1.23
SL11a	3.51

When acting against maltose, the α-glucosidases of the particular rhizobial strains followed the same activity pattern as against *O*-NP-α-D-gluco pyranoside: SL9b, SL3 and SL11a showed a high activity while the activities of SL4, SL10 and SL7b were relatively low. Figure 3.2 compares specific activities against the nitrophenyl glycoside and maltose. Maltose (4-*O*-α-D-glucopyranosyl-D-glucose) molecules have a specific α-linkage, i.e. α(1,4) bond, while the α-bond in O-nitrophenyl-α-D-glucopyranoside is not a specific one, which, therefore, can be hydrolysed by any α-glucosidase. For all the strains, α-glucosidase activity against nitrophenyl glucoside was higher than that against maltose indicating the presence of other α-glucosidases in addition to specific α-(1,4)-glucosidase.

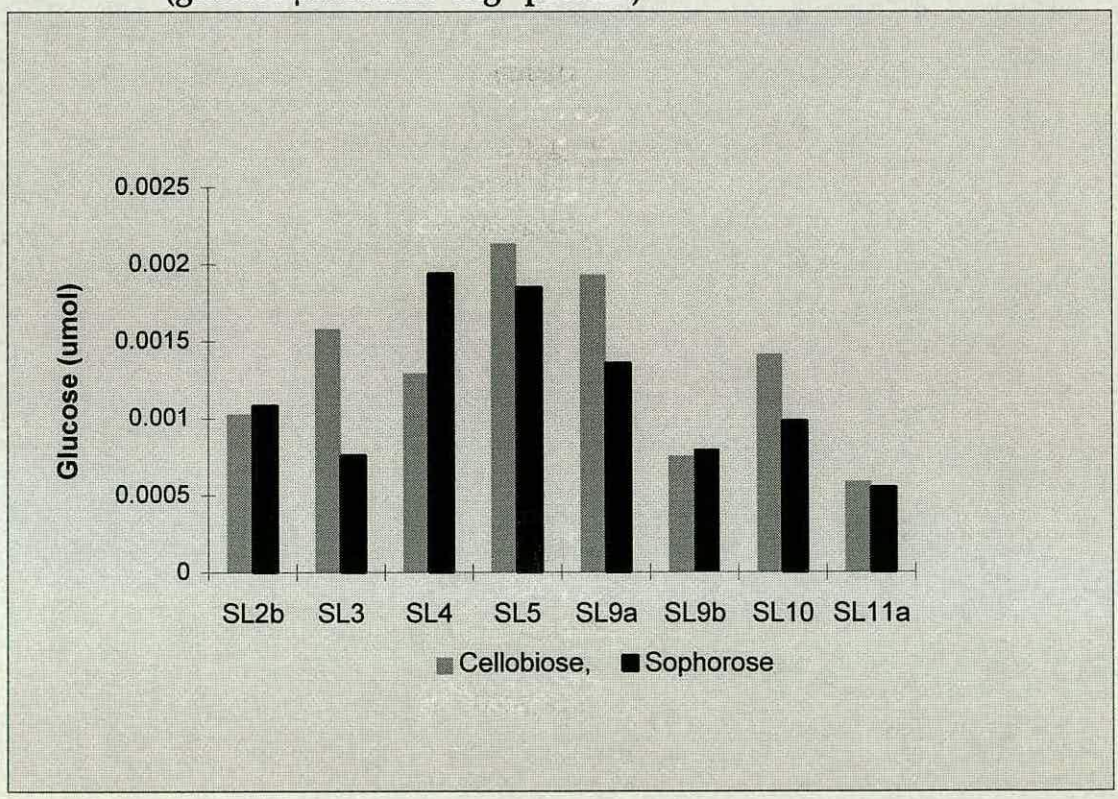
Figure 3.2. A comparison of specific activities against NP-glycoside and maltose (μmol $min^{-1}mg^{-1}$ protein)



Three substrates, namely cellobiose, gentibiose and sophorose were tested as the substrates for β-glucosidase activity of the strains which were positive

against O-nitrophenyl-β-D-glucopyranoside. Cellobiose (4-*O*-β-D-gluco pyranosyl-D-glucose) has β-(1,4) linkages while gentibiose (6-*O*-β-D-gluco pyranosyl-D-glucose) and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) carry β-(1,6) and β-(1,2) linkages respectively. None of the strains showed any significant activity against gentibiose indicating the absence of specific β-(1,6)-glucosidase in significant amounts. However, they were able to release glucose from both cellobiose and sophorose, mostly in similar amounts (Figure 3.3). One noticeable exception was SL3, which strongly preferred cellobiose to sophorose as the substrate. From these results, it can be concluded that the strains SL2b, SL4, SL5, SL9a, SL9b, SL10 and SL11a possess β-(1,4)- and β-(1,2)-glucosidases in similar amounts while SL3 has β-(1,4)-glucosidase about twise as much as β-(1,2)-glucosidase.

Figure 3.3. β-(1,4)- and β-(1,2)-glucosidase specific activities (glucose μmol $min^{-1}mg^{-1}$ protein)

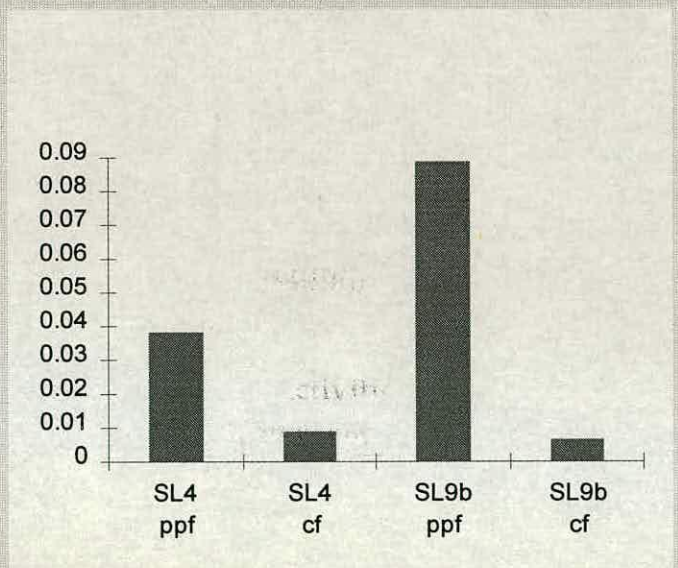


3.2.1. Location of glycosidases

Abe and Higashi (1982) reported the main location of β-glucosidase and β-galactosidase of *Rhizobium trifolii* 4S as the periplasmic space. When compared with the cell homogenate, they found that the glycosidase activities of the crude extracts of periplasm were significantly higher than that of the cell homogenate, indicating the periplasmic location of the particular enzymes.

In the present study, the periplasmic space contents of two Sri Lankan rhizobia, SL4 and SL9b, were separated from the cell-interior contents by the chloroform-treatment method and were used separately in α-glucosidase assay. A comparison is shown in figure 3.4.

Figure 3.4. α-glucosidase specific activities of the periplasm and the cytoplasm (μmol min^{-1} mg^{-1} protein)



ppf= periplasmic fraction, cf= cytoplasmic fraction

The α-glucosidase specific activity of the periplasmic fraction of the strain SL4 was 0.038μmol min^{-1} mg^{-1} protein and that was about 4.5 times higher than that of the cytoplasmic fraction indicating that α-glucosidase is mainly located in the periplasmic space. The α-glucosidase activities of the periplasmic and cytoplasmic fractions of SL9b showed a wider difference; The α-glucosidase specific activity of the periplasmic fraction was 0.089μmol min^{-1} mg^{-1} protein and that was about 14 times higher than that of the cytoplasmic fraction. These results clearly indicated the periplasmic locality of α-glucosidase in SL4 and SL9b.

3.3. Starch as a carbon source

Dextrins are polysaccharides of intermediate chain length formed from starch components by the action of amylases. Jordan (1984) indicated that dextrins are rarely used by rhizobia. Idrissi *et al.* (1996) reported that three out of twelve rhizobial strains isolated from *Ceratonia siliqua* (carob tree) nodules showed amylolytic activity. Apart from these citations, the ability of rhizobia to hydrolyse starch, which is the common storage polymer of plants and therefore could be a good carbon source for the symbiont, was not reported earlier. Hence, in the present study, it was regarded as another significant aspect that should be investigated.

Among the rhizobial strains studied, two Sri Lankan strains, namely SL9b and SL4 showed strong starch hydrolytic activities. Within a short period of growth (24-36h at 30°C), they gave large clear zones for the iodine test on starch-agar plates, indicating the presence of amylase. The cell lysates and the external growth medium of both strains were strongly-positive for starch-hydrolysis tests. In order to purify the intracellular and extracellular amylases of both rhizobial strains the following sequential procedure, i.e., ammonium sulphate precipitation, ion-exchange chromatography, affinity chromatography and gel-electrophoresis, was followed. Amylase activities of different samples obtained in the study were detected by a new iodine method described in chapter 2.3.5 (p. 106). The conventional method of adding few drops of a sample onto a starch-agar plate, flooding it with

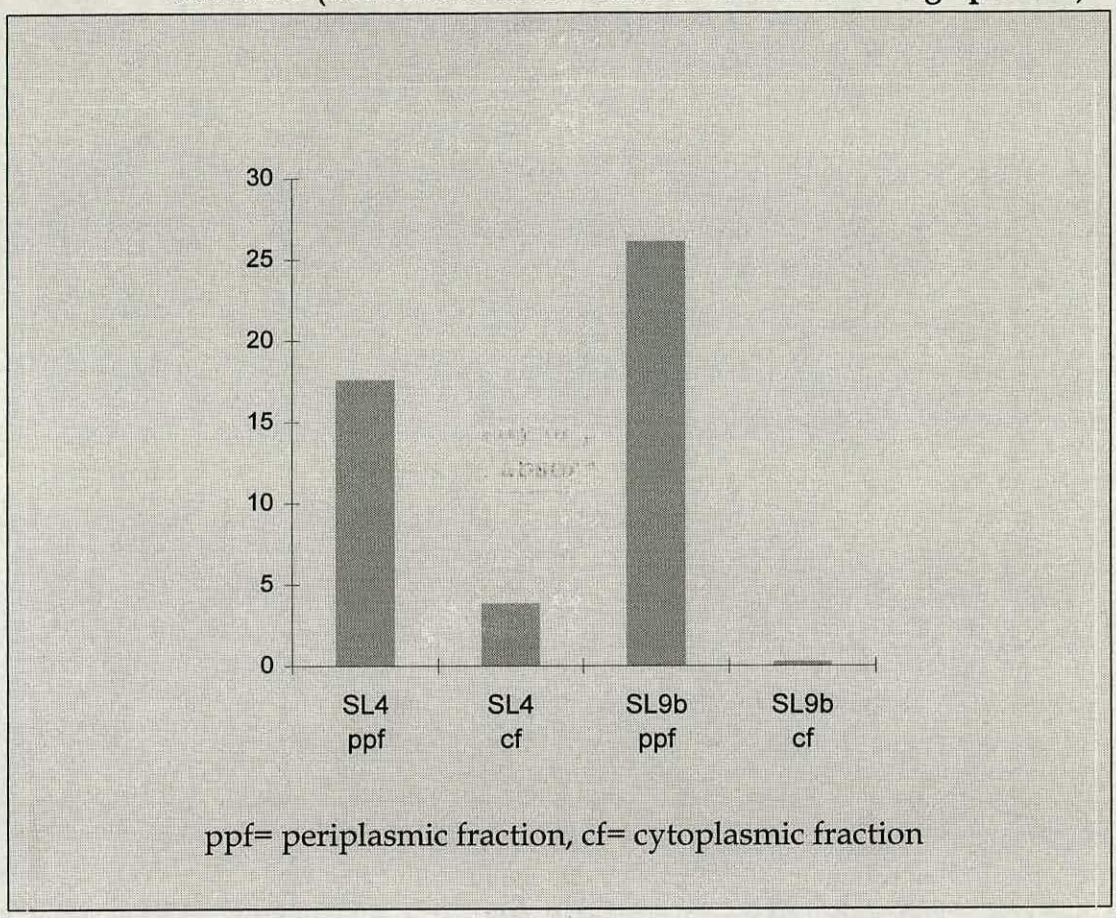
iodine after a period of incubation and regarding a clear zone as an indication for the presence of amylase in the sample was proven as giving wrong results. For instance, samples of water could make clear zones on starch-agar plates, possibly by washing-off starch. The new method was also used conveniently to estimate the amylase specific activity of different samples which was expressed as the decrease of absorbace of blue colour at 650nm min^{-1} mg^{-1} protein under specified temperature and pH. In addition, the release of glucose and reducing sugar from starch were used in some experiments to estimate the amylase specific activity.

3.3.1. Intracellular amylases of SL4 and SL9b

3.3.1.1. Location of the enzyme

The periplasmic and cytoplasmic fractions of both strains were separated by the chloroform-treatment method. The amylase specific activity of each fraction was estimated and the results clearly revealed the periplasmic location of the enzyme (Figure 3.5). In the strain SL4 the periplasm contained about 82% of the total intracellular amylase specific activity while in SL9b it was 98%.

Figure 3.5. Amylase specific activity of periplasm and cytoplasm of SL4 and SL9b (decrease of absorbance at 650nm min^{-1} mg^{-1} protein)



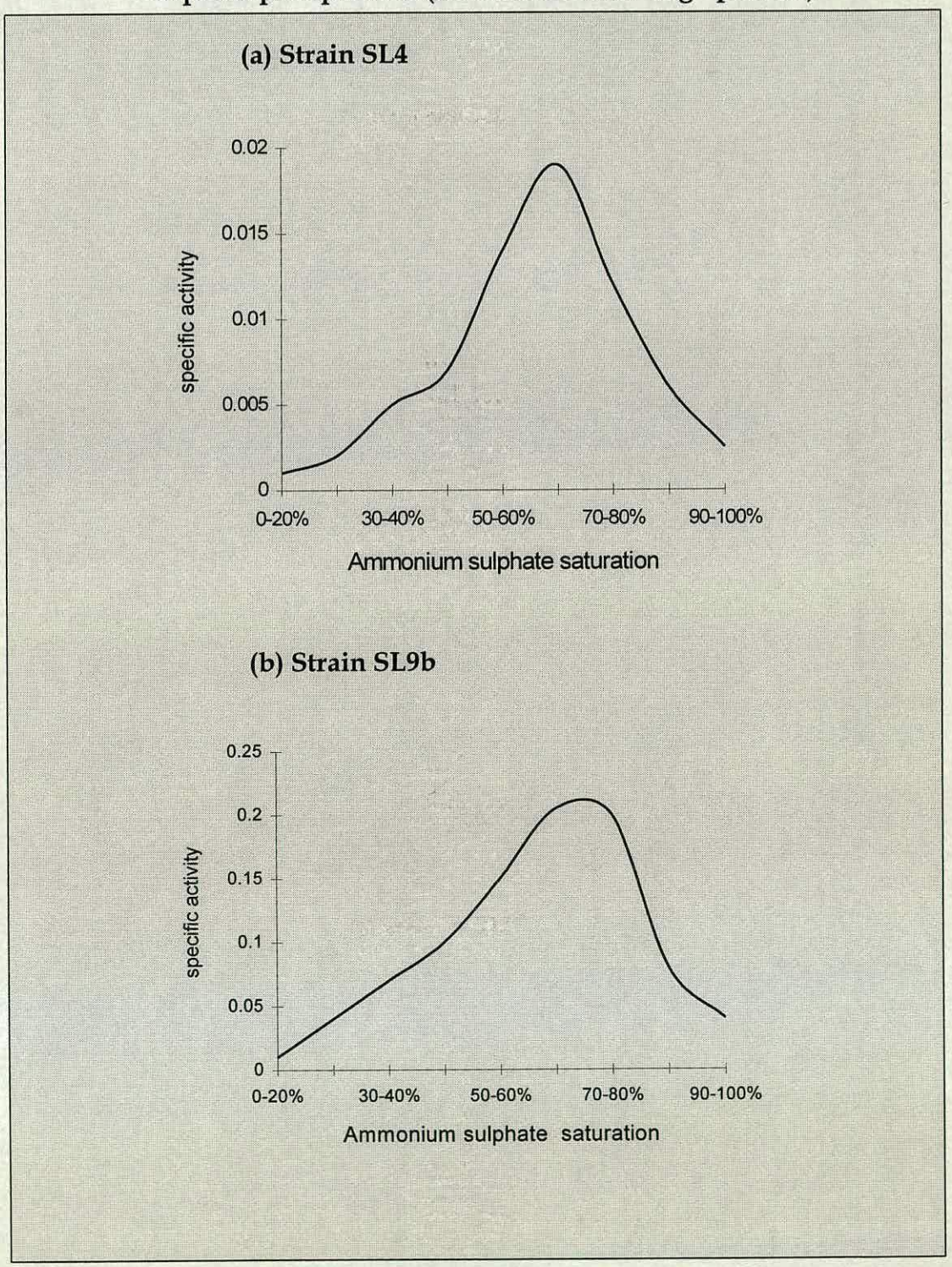
3.3.1.2. Purification

Ammonium sulphate precipitation (salting-out)

The amylase active range of the ammonium sulphate precipitation of the cell lysate of SL4 was the precipitates of ammonium sulphate saturation between 50-80%. Figure 3.6a shows the amylase activities of different ammonium sulphate precipitates of SL4. The precipitate of 60-80% saturation, which had the highest amylase activity, was chosen to run in the subsequent anion exchange chromatography.

The intracellular amylase of the strain SL9b was very similar to that of SL4 in the ammonium sulphate precipitation step. The precipitate of ammonium sulphate saturation 55-80% was the most active fraction (Figure 3.6b)

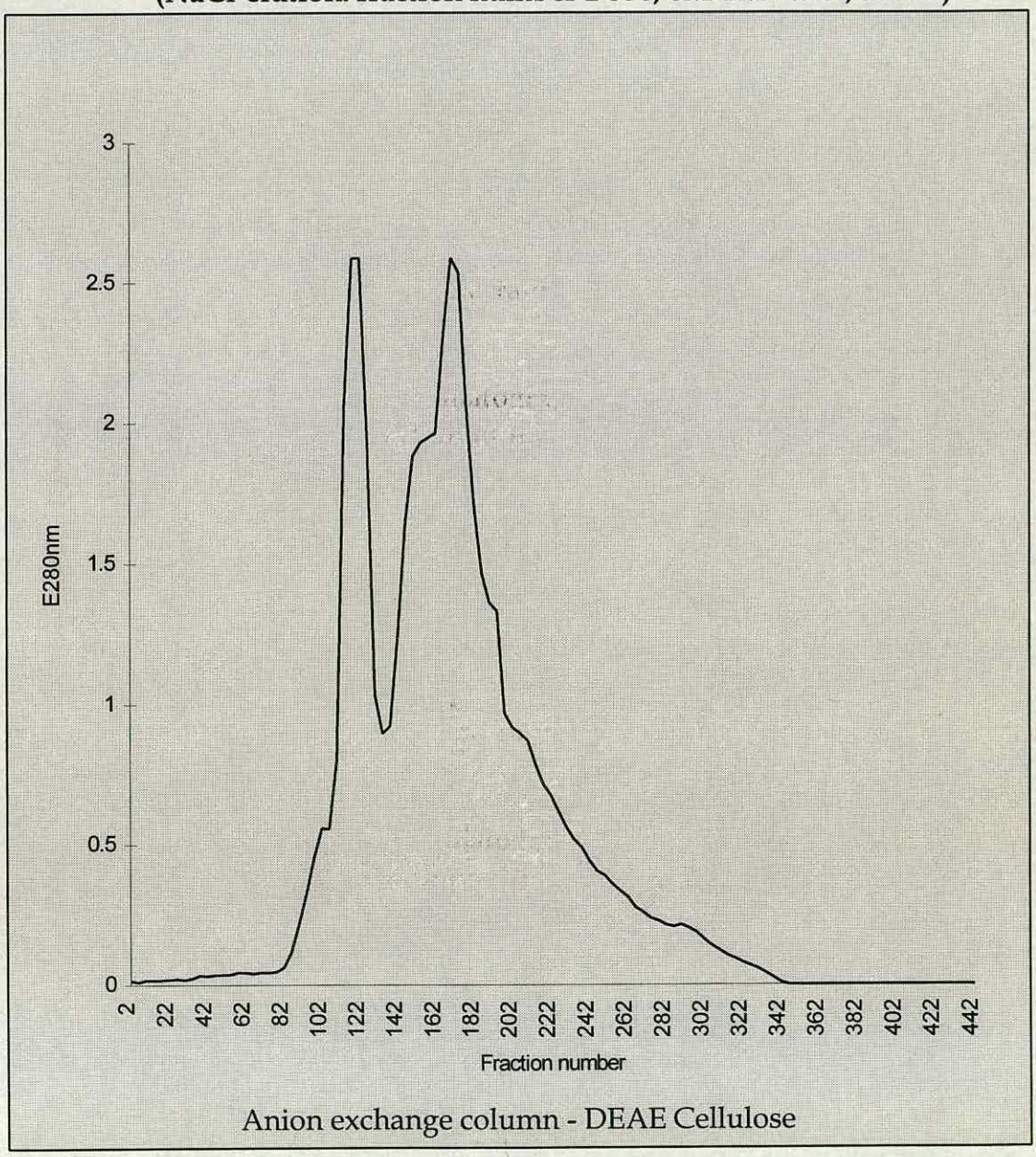
Figure 3.6. Amylase specific activities of diffrent fractions of ammonium sulphate precipitation (absorbance min^{-1} mg^{-1} protein)



Ion exchange chromatography

DEAE-cellulose columns (4.52cm^2 x 50cm) at pH7.0 were used in anion exchange chromatography at flow rates between 30-45ml h^{-1}cm^{-2}. The SL4

Figure 3.7. Anion exchange chromatography: SL4 Elution profile (NaCl-elution: fraction number 1-350, 0M-1M NaCl, 500ml)

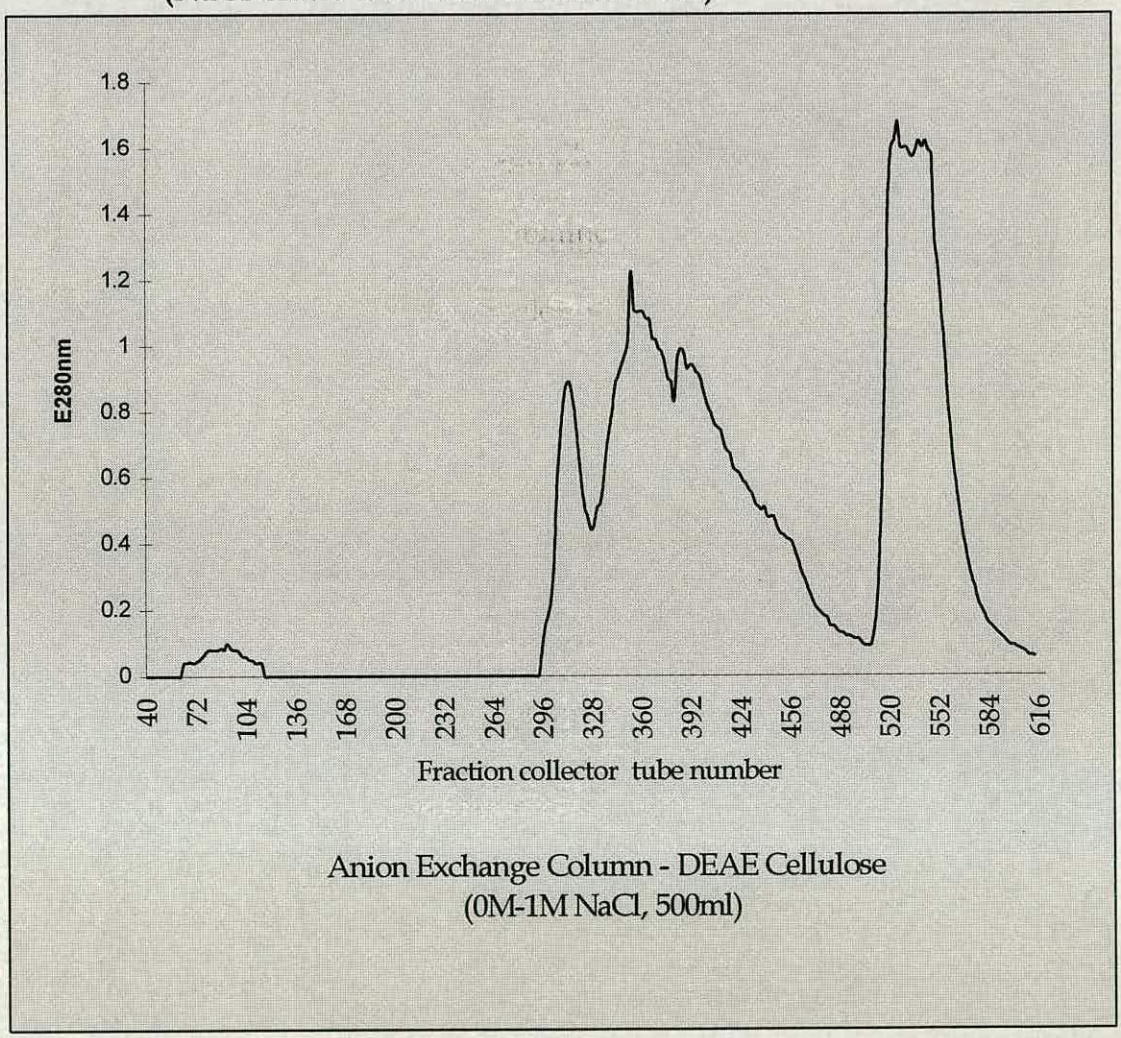


intracellular, 60-80% $(NH_4)_2SO_4$ fraction gave three separate fractions. The unadsorbed fraction (A-IEC1) which carried a high concentration of proteins

was amylase negative or showed a very little activity indicating that the target enzyme was adsorbed to the column. The subsequent elution profile obtained by applying a gradient of 0-1M NaCl 500ml in pH7, 20mM Tris buffer gave two peaks (Figure 3.7). The pooled fractions under the second peak area was amylase-positive

The same ion-exchange chromatography procedure was followed for the strain SL9b and the elution profile obtained (Figure 3.8) was basically similar to that of SL4. It also had two major peaks eluted by NaCl, of which the

Figure 3.8. Anion exchange chromatography: SL9b Elution profile (NaCl-elution: fraction number 1-600)



the second one, A-IEC3, contained the amylase activity. When compared with the SL4 elution, one noticeable different was the higher NaCl strength needed for the SL9b amylase to be eluted, and it indicated that the anionic charge of SL9b amylase is larger than that of SL4 .

Affinity chromatography.

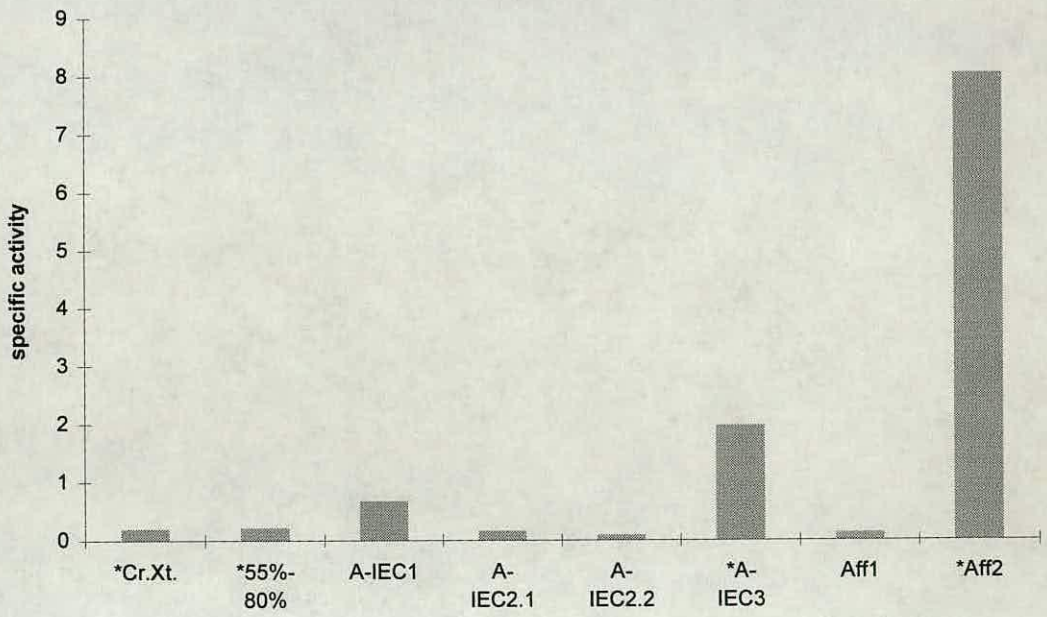
Two types of affinity columns, namely glucose-agarose and maltose-agarose, were used for further purification of amylase containing fractions obtained from ion exchange chromatography. The fractions of both SL4 and SL9b gave single peaks under gradient elution with 0-1M NaCl 500ml in pH7, 20mM Tris buffer. Maltose and glucose affinity columns were not very different in efficiency except the fact that the former allowed a better flow-rate. The non-adsorbed fractions of the affinity chromatography of both strains were had only traces of amylase.

The efficiency of the purification process

The amylase specific activities of different fractions obtained in each step of purification are compared in order to measure the efficiency of purification. The figure 3.9 shows the increase of amylase specific activity during the purification of SL9b, in terms of decrease of absorbance at 650nm min^{-1} mg^{-1} protein. The amylase specific activity of the initial crude cell lysate, which

was 0.20 min^{-1} mg^{-1} protein, increased up to 0.21 min^{-1} mg^{-1} protein, i.e., 1.05 fold increase in the first step of purification, i.e., ammonium sulphate precipitation. The amylase specific activity of the amylase-containing fraction of anion exchange chromatography (A-IEC3), 0.96 min^{-1} mg^{-1} protein, was a 21 fold increase from that of the crude lysate. The most-purified fraction of the purification process, Aff2, which was obtained by affinity chromatography, showed an amylase specific activity as high as 8.04 min^{-1} mg^{-1} protein, i.e., 64 fold increase from that of the initial crude cell lysate.

Figure 3.9. Amylase specific activities of different fractions obtained in the purification of SL9b intracellular amylase (decrease of absorbance min^{-1} mg^{-1} protein)

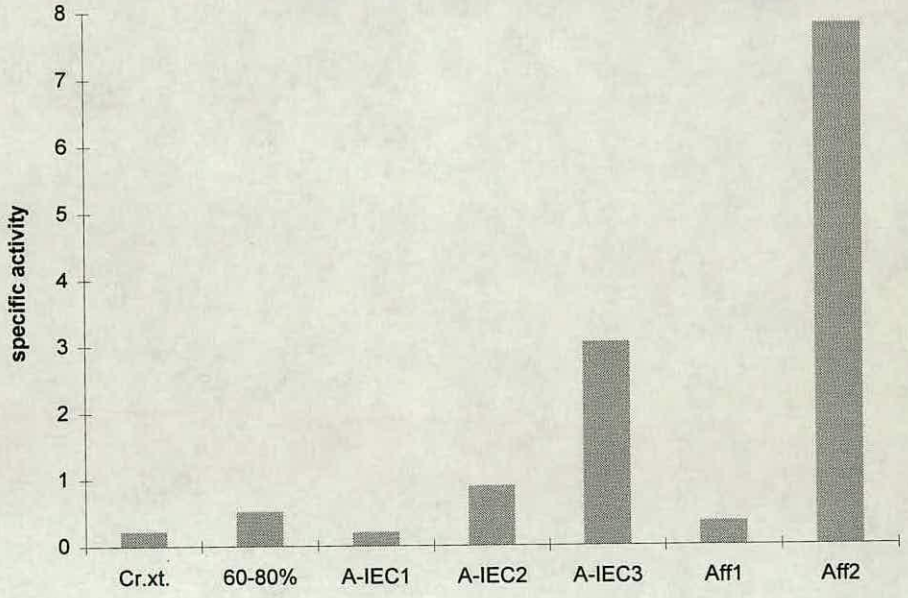


** The fractions selected in each step of purification*

Cr. Xt. = crude cell lysate, A-IEC = Anion exchange chromatography (numbers indicate the consecutive peaks in the elution profile)
Aff = Affinity chromatography, 55-80% = $(NH_4)SO_4$ saturation

Figure 3.10 shows the efficiency of the purification process in the case of SL4 intracellular amylase. There was a 2.3 fold increase of specific activity in the ammonium sulphate purification, i.e., in terms of decrease of absorbance at 650nm min^{-1} mg^{-1} protein, the specific activity increased from 0.22 to 0.52. The most amylase active sample obtained from the ion exchange chromatography showed 13.6 fold increase in amylase specific activity when compared to that of the initial crude cell lysate. The final step of purification, affinity chromatography achieved a 34.8 fold increase in the specific activity.

Figure 3.10. Amylase specific activities of different fractions obtained in the purification of SL4 intracellular amylase (decrease of absorbance min^{-1} mg^{-1} protein)



** The fractions selected in each step of purification*

Cr. Xt. = crude cell lysate, A-IEC = Anion exchange chromatography (numbers indicate the consecutive peaks in the elution profile)
Aff = Affinity chromatography, 60-80% = $(NH_4)SO_4$ saturation

3.3.1.3. Gel-electrophoresis and molecular weight determination

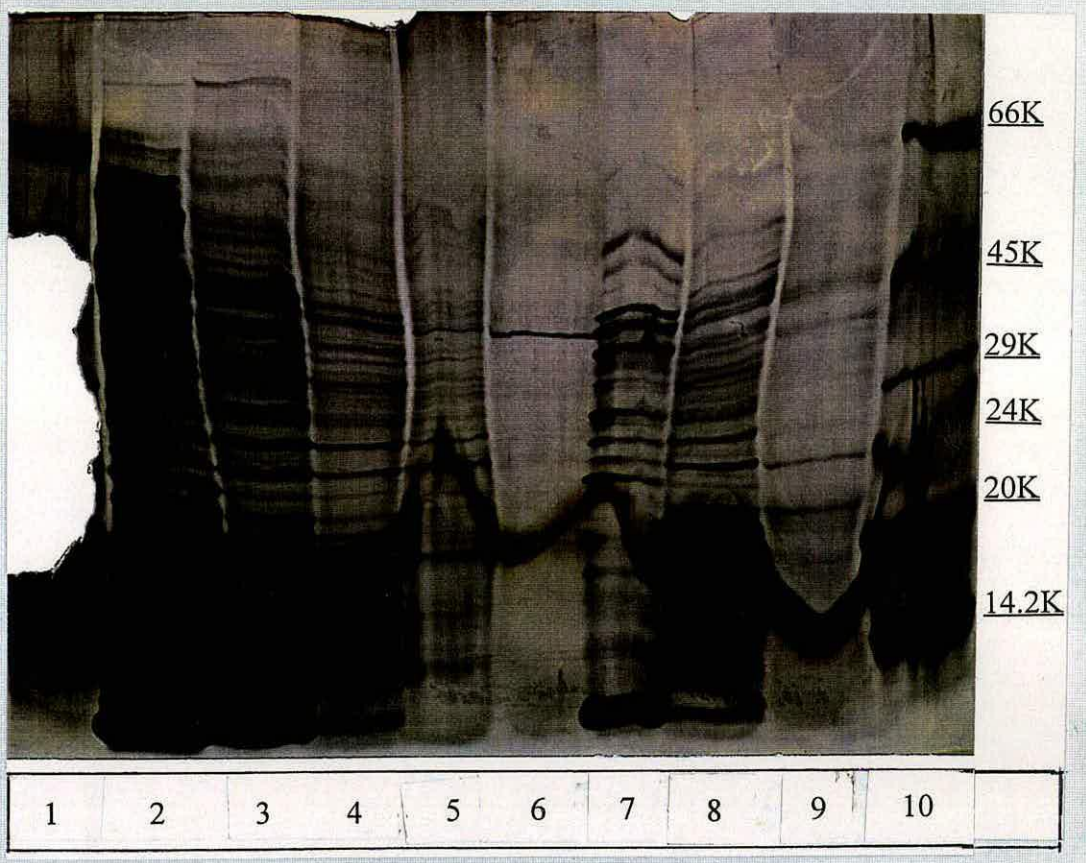
Polyacrylamide gel electrophoresis was carried out for the amylase-active fraction obtained from affinity chromatography. For comparison, other amylase-containing fractions were also run parallel with affinity chromatography fractions. The standards used were bovine serum albumin (66kDa), ovalbumin (45kDa), carboxy anhydrase (29kDa), trypsinogen (24kDa), trypsin inhibitor (20kDa) and lactalbumin (14.4kDa). The silver stain method was the method used in staining the gels. Figure 3.11 shows the stained gel of SL4 intracellular amylase.

The distances which the tracking dye, the standard proteins and the unknown amylases travelled in the gels were used in the following method in order to estimate the molecular weights of the amylases. Rf value for each protein was calculated using the formula:

Rf= the distance of protein travelled/ the distance of the tracking dye travelled

In order to derive the molecular weight of the intracellular amylase of strain SL4, the molecular weights of the standard proteins were plotted against their Rf values. The Rf value of the unknown protein was determined using the distance it travelled in the gel and that value was used with the curve to calculate the molecular weight of the amylase. According to the calculation, the molecular weight of the intracellular amylase of SL4 was approximately 33kDa.

Figure 3.11. The electrophoresis gel of SL4 intracellular amylase



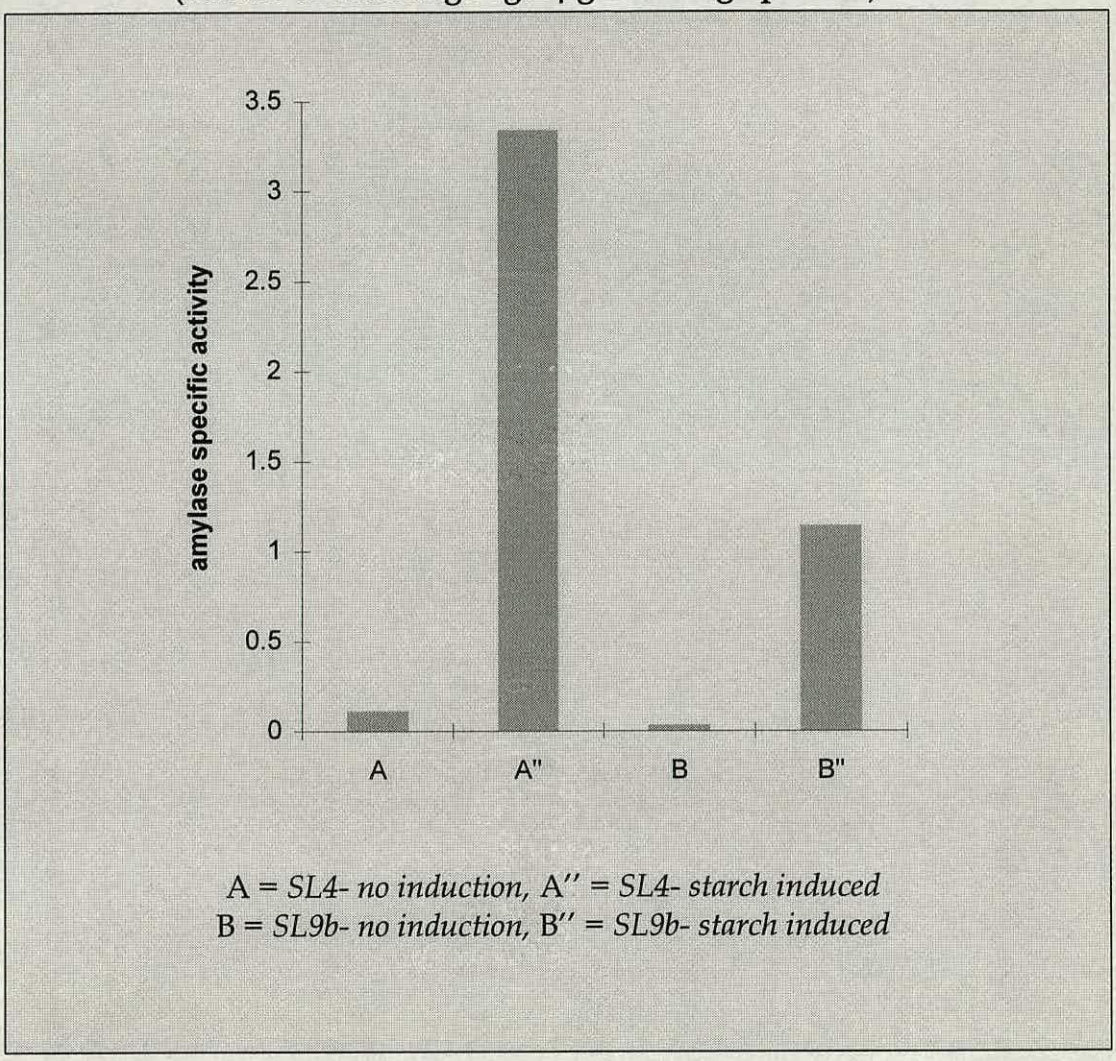
1 and 10 = Standars (Bovine serum albumin -66K, Ovalbumin -45K, Carbonic anhydrase -29K, Trypsinogenase- 24K, Tripsin inhibitor- 20K and Lactalbumin- 14.2K)
2 = Ammonium sulphate fraction 60%-80%
3 = Anion exchange fraction
4= Glucose affinity fraction 1
5 = Glucose affinity fraction 2
6 = Glucose affinity fraction 3
7 = Maltose affinity fraction 1
8 = Maltose affinity fraction 2
9 = Maltose affinity fraction 3

3.3.2. Extracellular amylases of SL4 and SL9b

3.3.2.1. Are they inducible?

The presence of extracellular amylases in SL4 and SL9b was detected when these two rhizobial strains, after a period of growth, gave clear zones for iodine test on starch-agar plates. Laboratory tests were carried out to establish whether these extracellular amylases were inducible by the substrate or not. The strains SL4 and SL9b were grown for 3d. at 30°C separately with and without 0.1% soluble starch in the medium. After growth, the cells were removed from the extracellular solutions by centrifugation. Tangential-flow filtration was employed as an attempt to concentrate and separate proteins from the rest of the solution (p. 94). The extracellular protein solutions were tested against starch at pH 7, 30°C, and, in order to estimate the hydrolysis of the substrate, reducing sugar tests were carried out for samples taken from the assay mixtures. The results, given in figure 3.12 clearly indicated that the extracellular amylases of SL4 and SL9b were inducible by starch. When grown with added starch in the medium, SL4 increased the extracellular amylase specific activity by about 29 fold compared to cultures grown without the substrate. Following the same trend, SL 9b showed approximately 34 fold increase in the extracellular amylase specific activity when grown in the presence of starch.

Figure 3.12. Extracellular amylase specific activities of SL4 and SL9b (release of reducing sugar μg min^{-1} mg^{-1} protein)



3.3.3..Analysis of starch hydrolysates

The hydrolysates of starch upon the activities of amylases of the strains SL4 and SL9b were analysed by the methods described in pp. 107-108. The paper chromatograms of the starch hydrolysates of SL4 intracellular and extracellular amylase activities are shown in figure 3.13 (p. 145). Both the intracellular and extracellular components were able to hydrolyse starch into

products of different molecular weights, ranging from glucose, maltose, maltotriose to longer oligosaccharides. Starch hydrolysate of SL9b intracellular amylase activity was run through gel-filtration column BioGel-P2 and the two fractions that eluted were run on paper chromatograms (figure 3.14, p. 146). The results indicated the presence of different products like those produced by SL4.

Figure 3.13. Starch hydrolysates of SL4

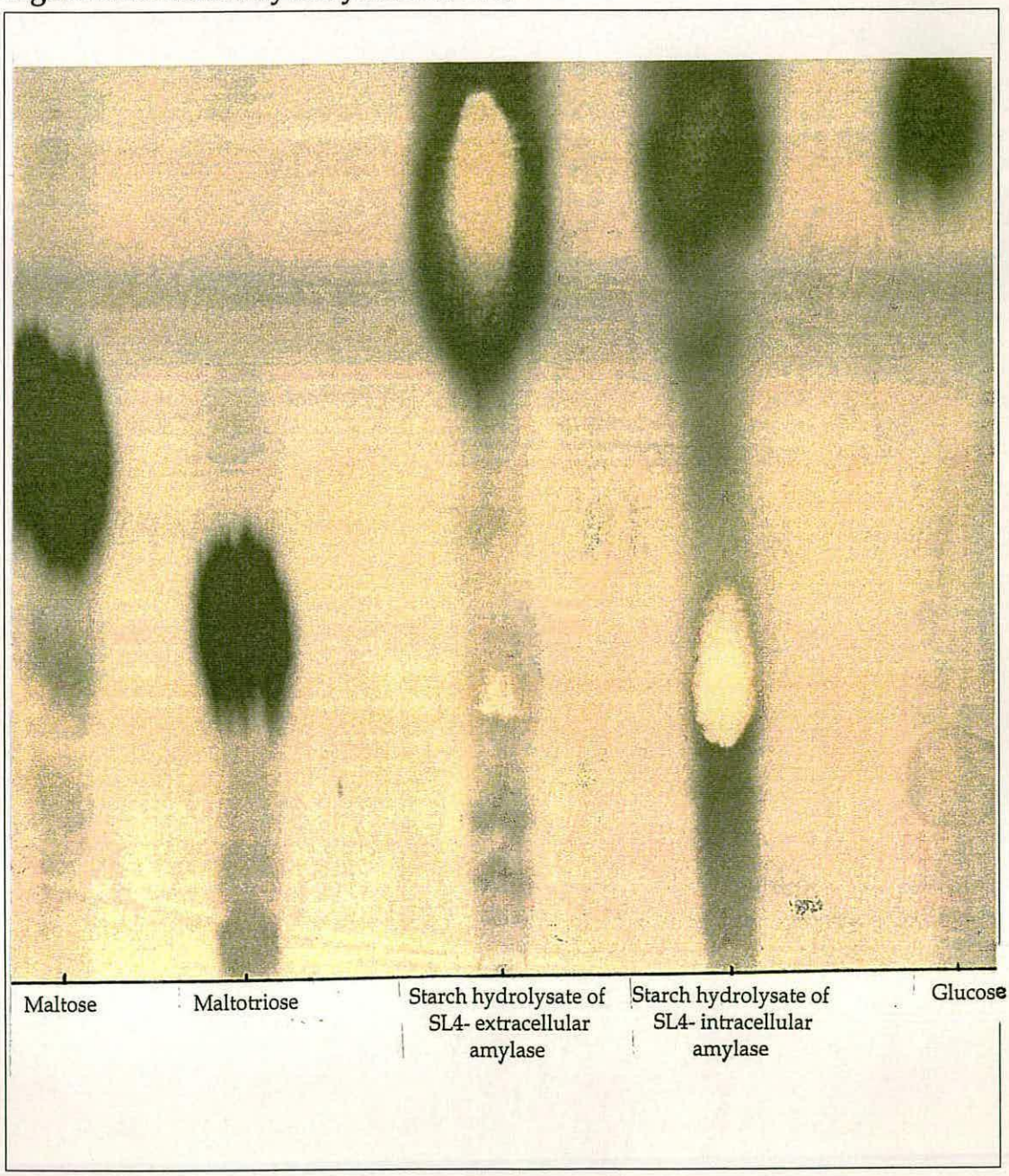


Figure 3.14. Paper chromatogram of P2 fractions- SL9b

3.4. Chemical and biochemical analysis of exopolysaccharides (A comparative study with known rhizobial exopolysaccharides)

Almost all the Sri Lankan rhizobial strains and the strains isolated from horticultural legumes in Scotland were extensive producers of exopolysaccharides so that it was possible to harvest high yields of exopolysaccharides from broth cultures. The only exceptions were the two Sri Lankan strains SL2a and SL12, in which the exopolysaccharide production was relatively low, and, therefore, the concentration in liquid media was not high enough for efficient harvesting. They were grown on solid media and the cultures were suspended in minimum amounts of water, blended and centrifuged to remove the cells. The exopolysaccharides were then harvested by the acetone precipitation method (p. 93).

Several methods and approaches were followed in order to elucidate the basic structure and composition of the exopolysaccharides. The main aim was to find out whether there are any significant structure-compositional differences between well-defined rhizobial exopolysaccharides and the hitherto unstudied rhizobial symbionts of the particular tropical legumes from Sri Lanka. In the following pages, the contribution of each method towards the understanding of exopolysaccharides is discussed followed by, in chapter-4 (conclusions), a summation of all the results to give a final picture of each polymer.

3.4.1. Detection and estimation of organic substituents

3.4.1.1. Chemical estimation of acylation

Acetyl groups attached to side chains of rhizobial exopolysaccharides by *O*-acetyl bonds are common organic substituents of these polymers. The acetyl content as %($^{w}/_{w}$) of exopolysaccharide was analysed by the method (p. 101) described by Hestrin (1949). The results, given in table 3.7, showed that all the exopolysaccharides possess acetyl groups, which was mostly less than 5%($^{w}/_{w}$) of the polymer. SL5, SL7c, SL12 and 4S had slightly more acetyl in their exopolysaccharides while SL2a had the highest amount, i.e., 8.85%.

Table 3.7. Acetyl contents of rhizobial exopolysaccharides

Rhizobial strains	%($^{w}/_{w}$) acetyl in exopolysaccharides
SL2a	8.85
SL2b	3.07
SL3	4.73
SL4	3.46
SL5	5.27
SL7b	2.80
SL7c	6.76
SL9a	4.03
SL9b	2.84
SL10	4.64
SL11a	2.00
SL12	5.97
WGR234	1.71
AWU237	4.77
4S	6.87

Pyruvate goups are another typical components in rhizobial exopolysaccharides and are normally ketal-linked. The results obtained by Sloneker and Orentas (1962) method (p. 102) of pyruvyl analysis are given in table 3.8. The contents ranged from values less than 1%($^{w}/_{w}$) exopolysaccharides upto about 5%. SL2a, which had the highest amount of acetyl groups, had only insignificant amount of pyruvyl groups. SL11a, WGR234, AWU237 and SL9b had little pyruvyl groups in their exopolysaccharides while the rest had about 3-5%.

Table 3.8. Pyruvyl content of rhizobial exopolysaccharides

Rhizobial strains	$\%^{w}/_{w}$ pyruvyl in exopolysaccharides
SL2a	0.02
SL2b	4.08
SL3	4.71
SL4	4.56
SL5	4.64
SL7b	4.24
SL7c	4.54
SL9a	3.92
SL9b	1.86
SL10	4.88
SL11a	0.52
SL12	3.31
WGR234	0.90
AWU237	0.89
4S	3.55

3.4.1.2. Detection of acylation by ^{1}H-NMR spectroscopy

Proton NMR spectroscopy is regarded as an excellent tool for component determination in organic extracts, mainly because of its relatively high sensitivity and the universal occurrence of protons in organic metabolites. One of the important parameters measured in NMR spectra, the chemical shift (δ) in ppm, which is a measure of chemical environment of a given nucleus and hence indicative of the types of chemical groups that are present in a molecule, was used in the present study to detect the acylation of exopolysaccharides. Acetate, pyruvate and succinate groups were identified by comparing the chemical shifts of ^{1}H NMR spectra of exopolysaccharides with the tyical chemical shifts of these groups, which can change within a narrow range under different pH and temperatures. Fan (1996), in a list of chemical shifts of a number of commonly occuring metabolites, indicated that chemical shift values for acetate were 1.92ppm and 2.1ppm at neutral and acidic pH respectively. The value given for pyruvate was 1.48ppm while it was 2.39ppm for succinate. Levery *et al.* (1991) reported that, before elevating the temperature up to 77°C, the chemical shifts of acetate and pyruvate groups of the EPSII of *Rhizobium meliloti* were 2.152ppm and 1.459ppm respectively. A glucuronan produced by a mutant of the same bacterium gave a signal at 2ppm for acetate groups (Robolt *et al.*, 1995). Matulova *et al.* (1994) analysed succinoglycan from different microbial sources including different *Rhizobium* spp. by ^{1}H NMR spectroscopy and reported the chemical shift values under neutral pH for pyruvate and

succinate groups were 1.48ppm and 2.60ppm respectively. At pH 3.5, the succinate signal shifted to 2.74ppm. Chouly *et al.* (1995) recognised acetate, pyruvate and succinate substituents at 2.15ppm, 1.46ppm and ~2.60ppm respectively in ^{1}H NMR spectra of succinoglycan from *Rhizobium* and *Agrobacterium* sp.

Figure 3.15 shows the ^{1}H NMR spectra of the rhizobial exopolysaccharides of the present study, which were obtained at 25°C and without any pH adjustment. The possible influence of any intrinsic acidic nature of the exopolysaccharides on the chemical shift values is, therefore, not excluded. The chemical shifts of prominant peaks occurred in the range of the typical chemical shift values of acetate, pyruvate and succinate are given in table 3.9. The ^{1}H NMR spectra of all the exopolysaccharides tested showed signals that indicated the presence of pyruvate groups, however, with much variation in the intensity. SL4, SL7c, SL10, SL12x, Rhi-A, Rhi-G and 4S had prominant peaks at 1.5ppm resembling pyruvate groups. The peaks occurred in that particular chemical shift value for the exopolysaccharides of the strains SL2b, SL5, SL7b, SL9a, SL9b and SL11a were very small indicating that the contribution of pyruvate groups in the macromolecule structure was only very little. SL3 and AWU237 exopolysaccharides had their pyruvate peaks slightly shifted to 1.45ppm and the peak of the latter was also of low intensity. The reference polymer succinoglycan showed the presence of pyruvate at 1.52ppm and that too was not a large peak. The only significant

shift from the typical pyruvate chemical shift was shown by the exopolysaccharide of the Sri Lankan strain SL2a which was at 1.2ppm.

Except SL2a which was on 1.91ppm, all the other exopolysaccharides showed a signal for acetate at 1.95ppm on ^{1}H NMR spectra. For the exopolysaccharides SL10, SL11a, Rhi-E and succinoglycan, the acetate peaks were not of high intensity while SL7b and SL9b showed very small peaks.

The chemical shift of succinate was present in all but SL7c, Rhi-A and AWU-237 exopolysaccharides. For succinoglycan, the signal was at 2.09ppm, for 4S at 2.15ppm and for SL2a, the succinate signal was at 2.2ppm. SL5 and Rhi-G were the only exopolyaccharides which showed very little peaks for succinoglycan.

Figure 3.15. ^{1}H NMR spectra of exopolysaccharides (*overleaf*)

(the relevant peaks are identified in table 3.9;

The peak at 3.7ppm in most of the profiles is unidentified)

Figure 3.15. ^{1}H NMR spectra of exopolysaccharides

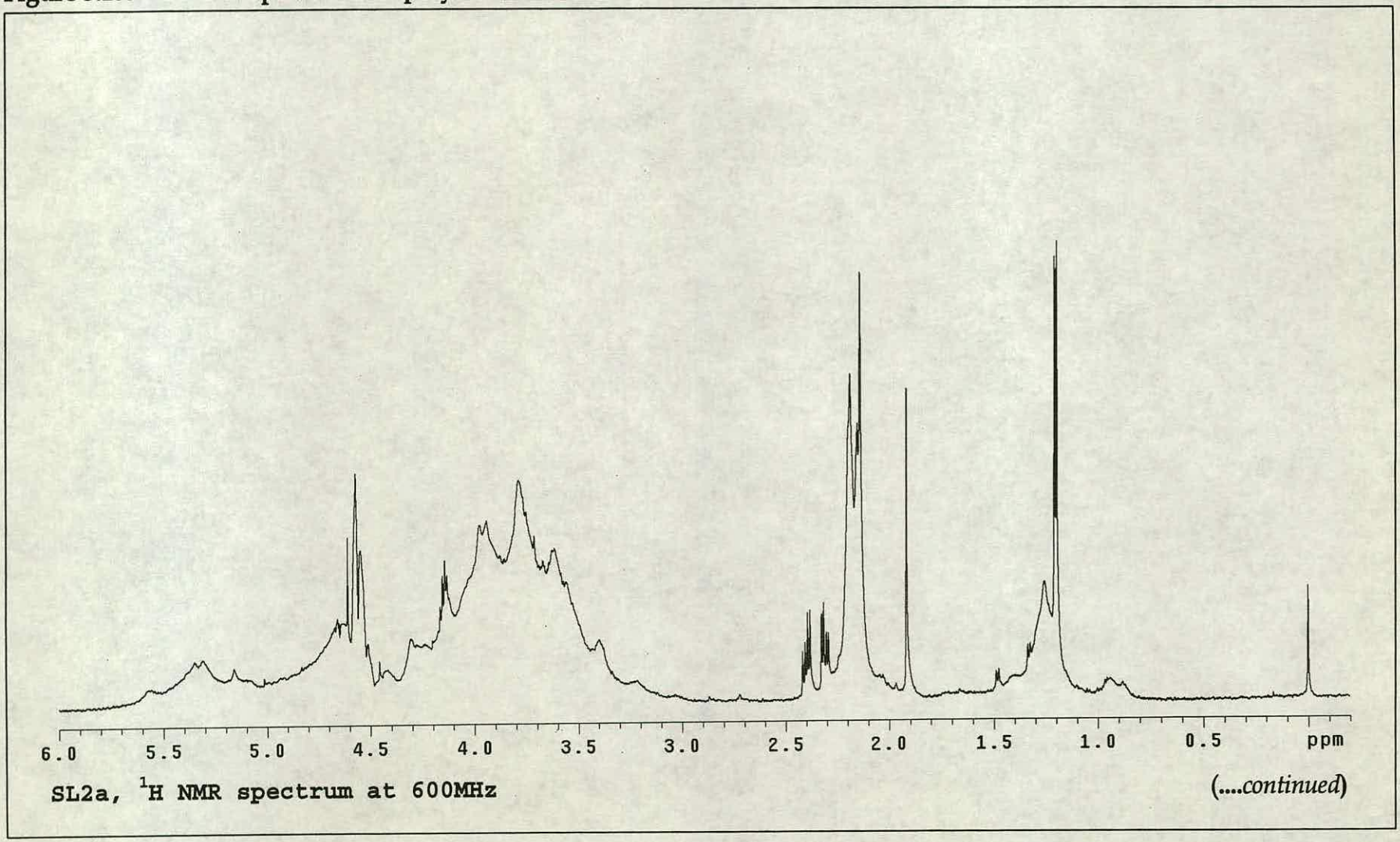


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Figure 3.15. (*...continued*)

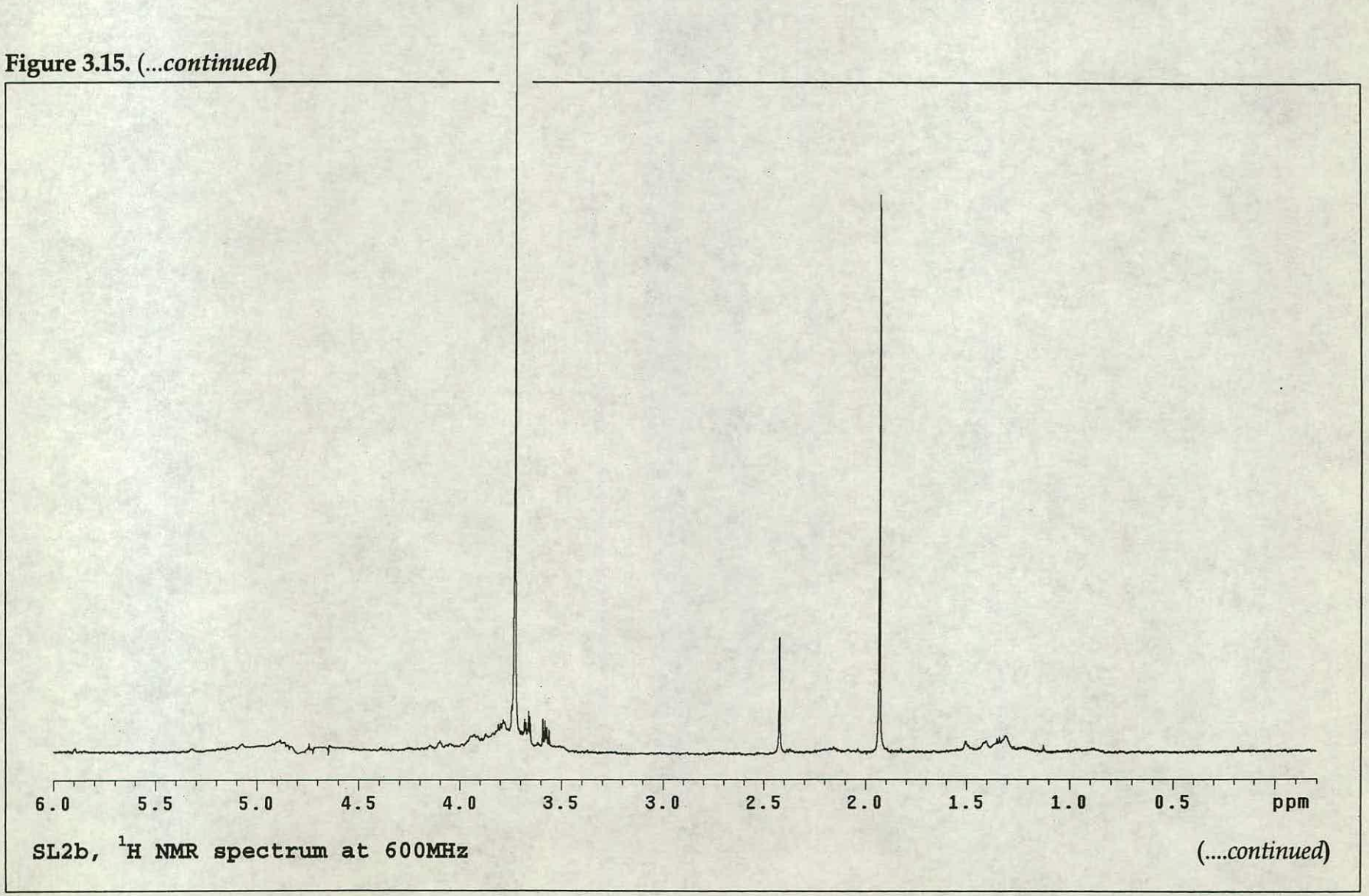

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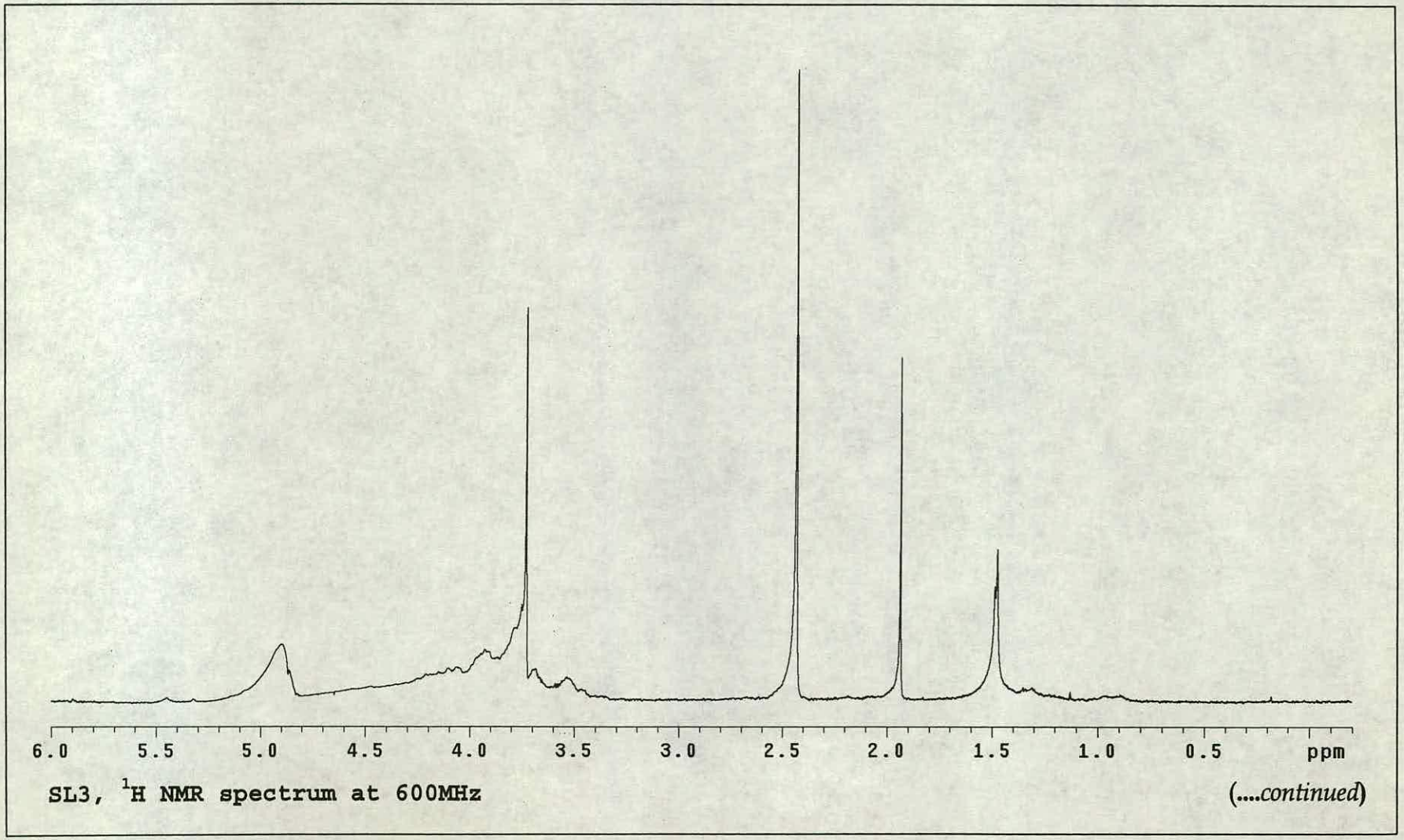
Figure 3.15. (*...continued*)



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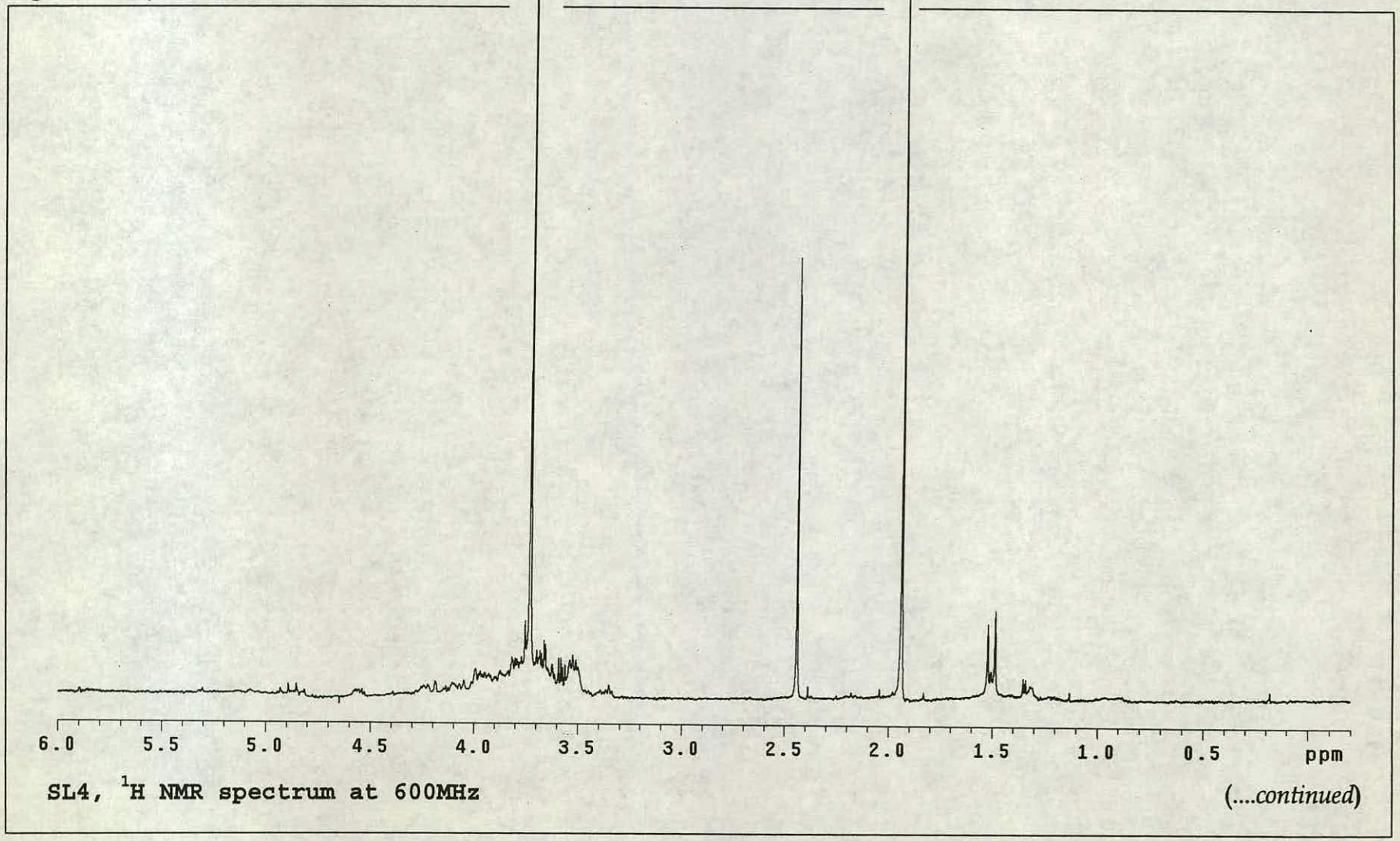

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Figure 3.15. (*...continued*)

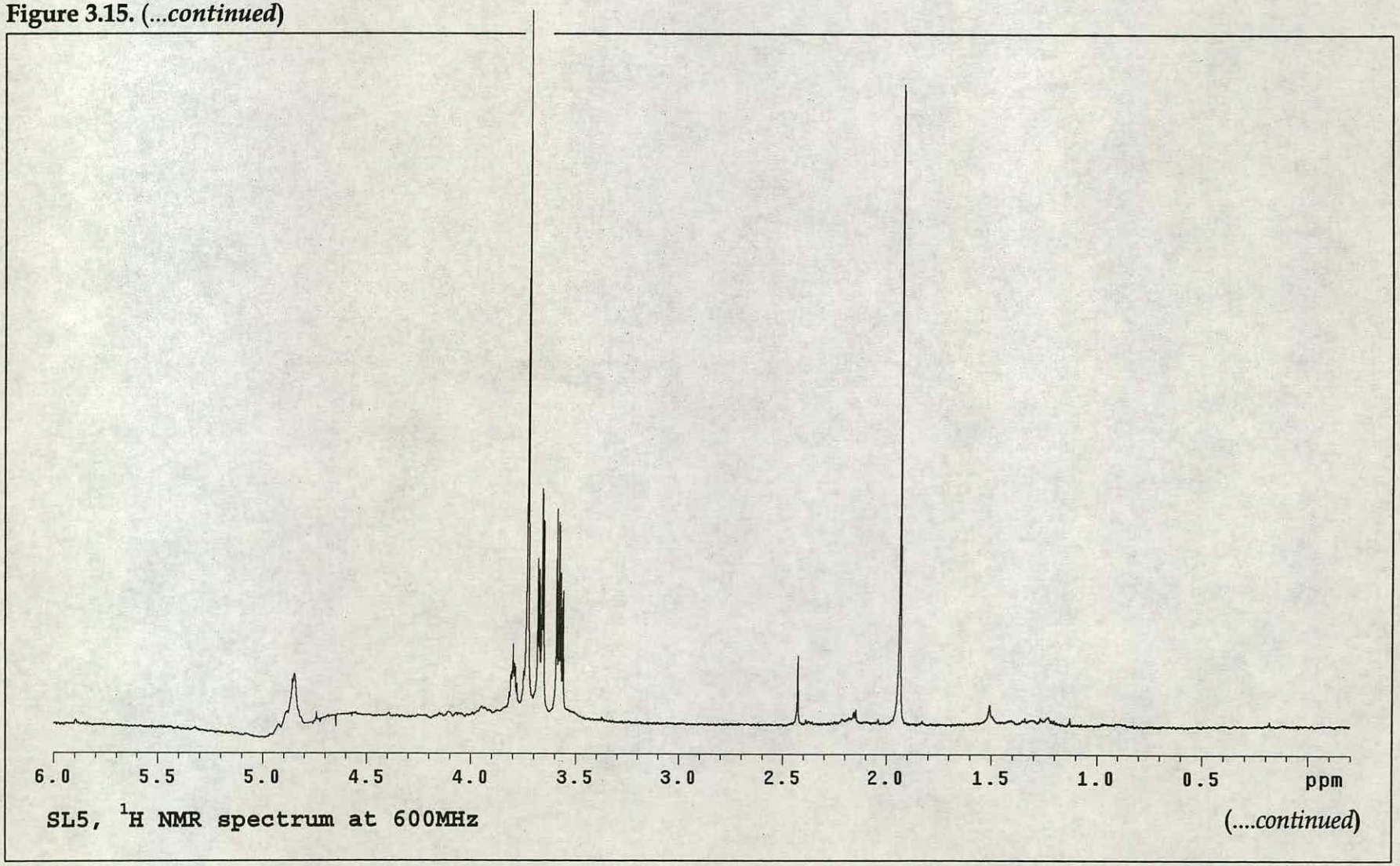

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Figure 3.15. (*...continued*)

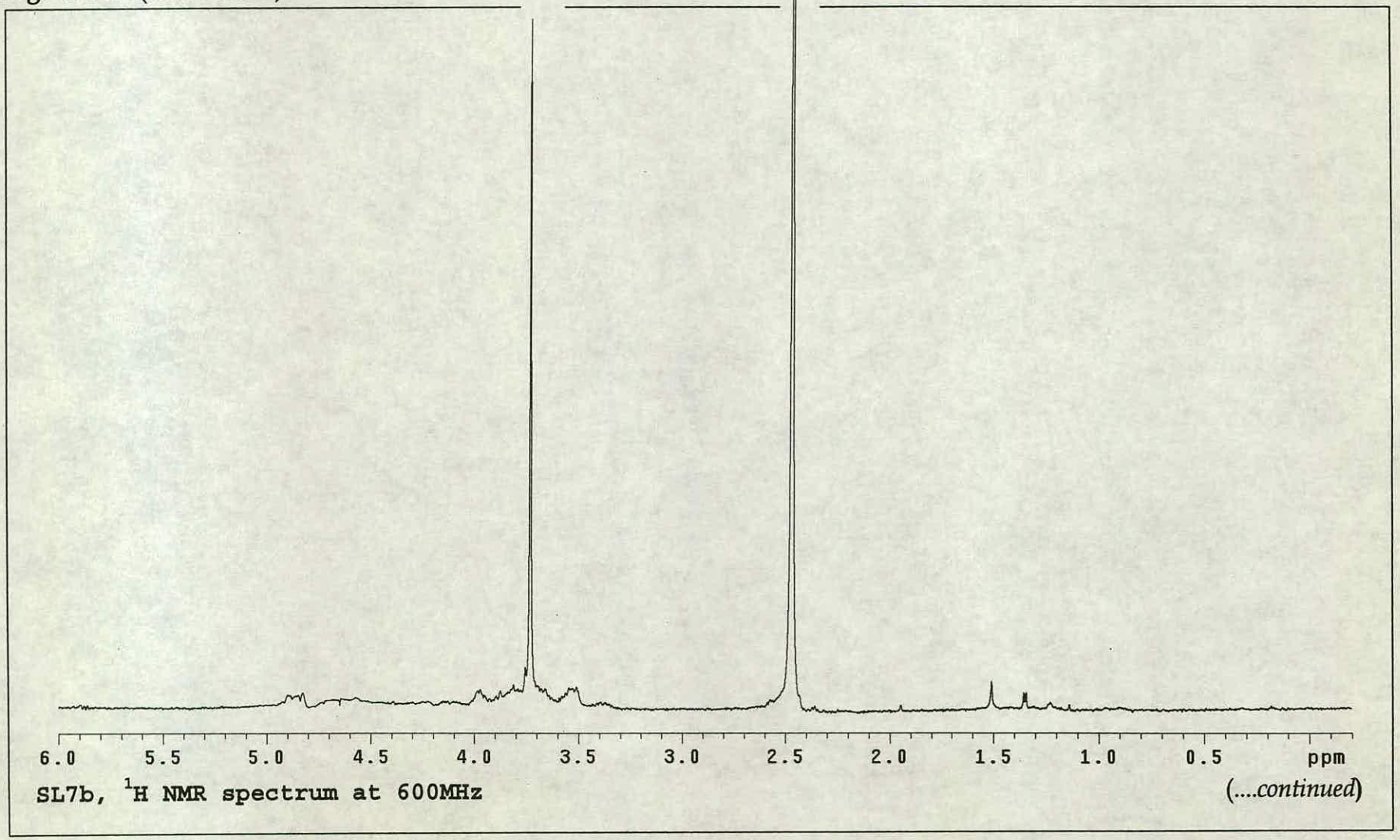

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Figure 3.15. (*...continued*)

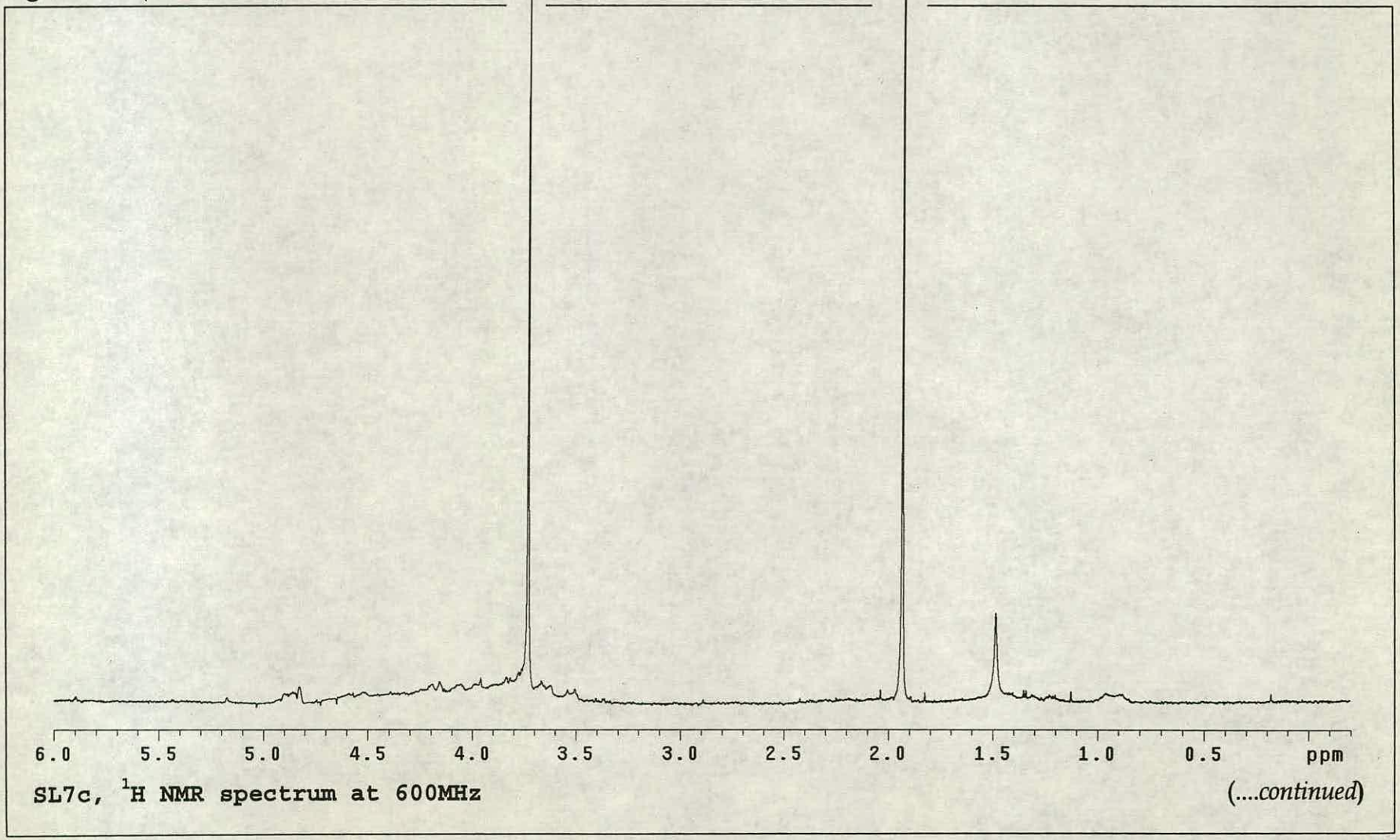

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Figure 3.15. (*...continued*)

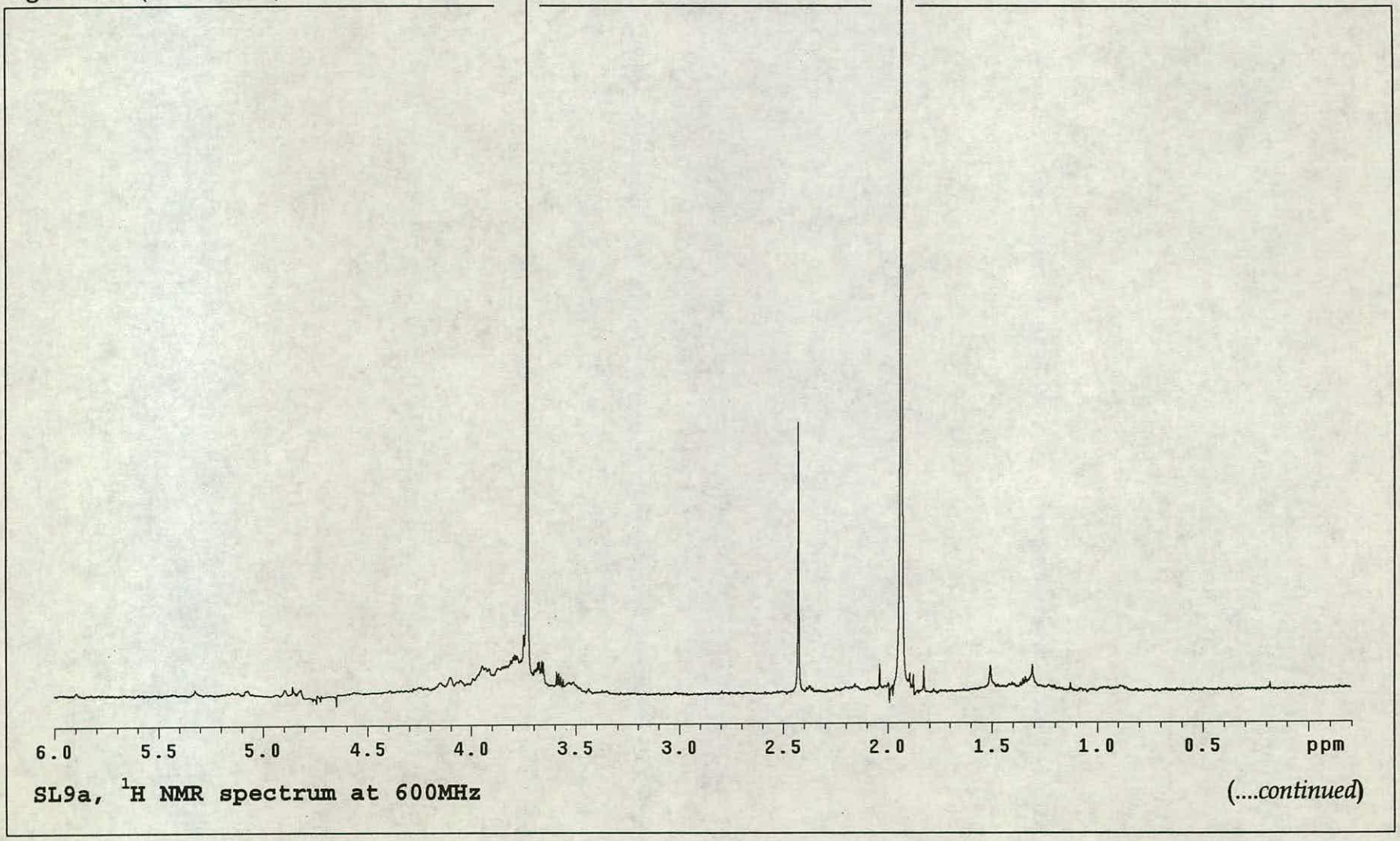

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Figure 3.15. (*...continued*)

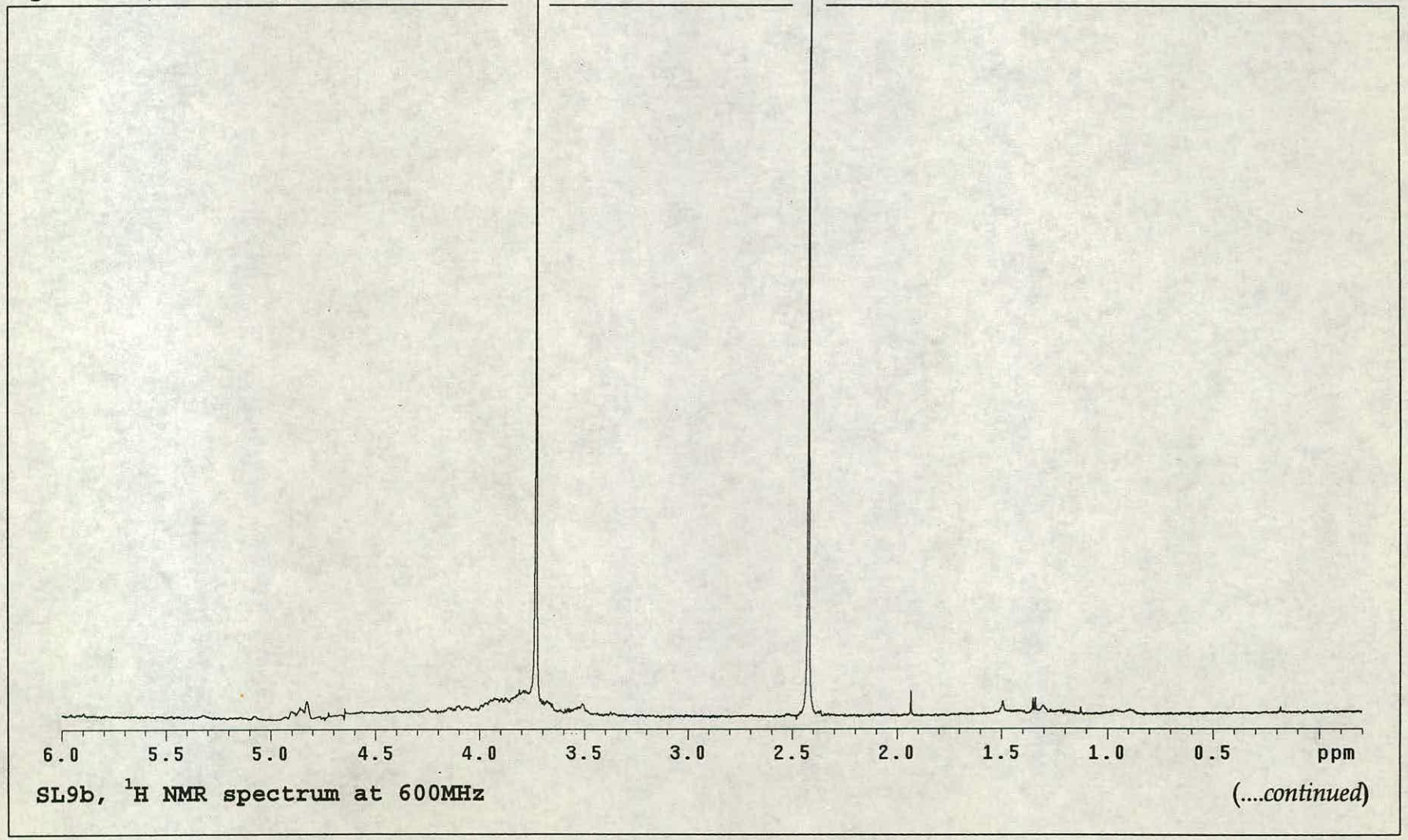

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Figure 3.15. (*...continued*)

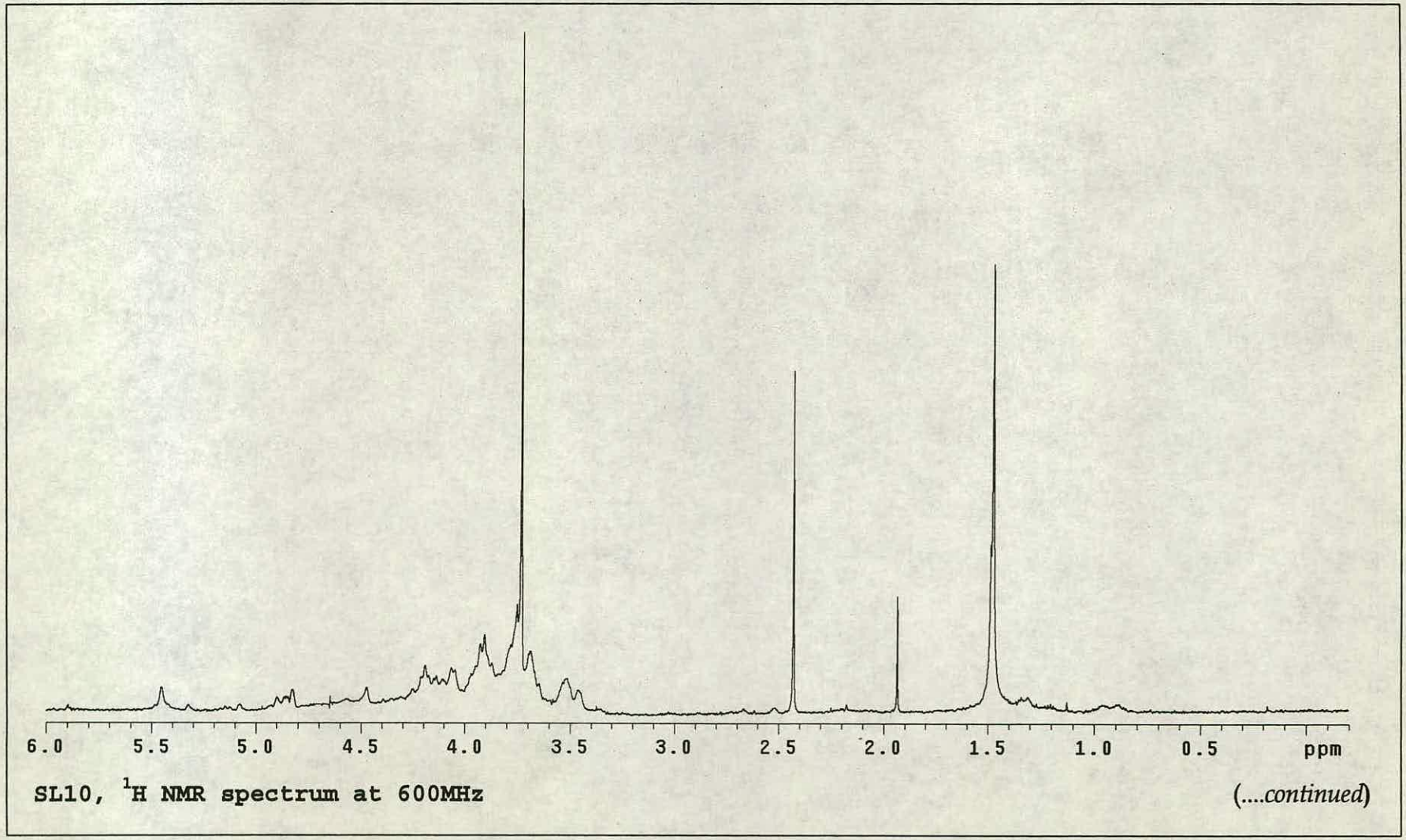

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Figure 3.15. (*...continued*)

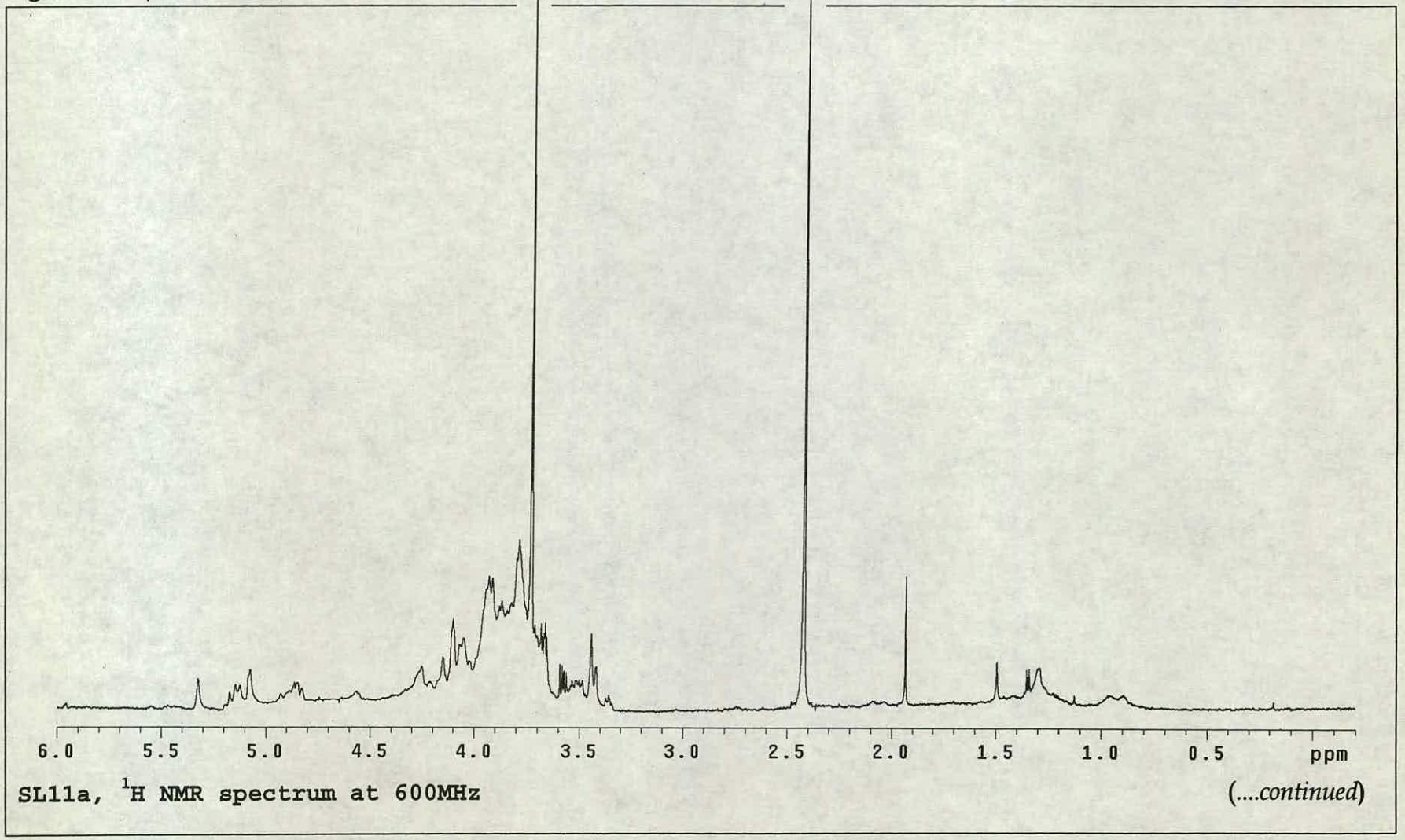

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Figure 3.15. (*...continued*)

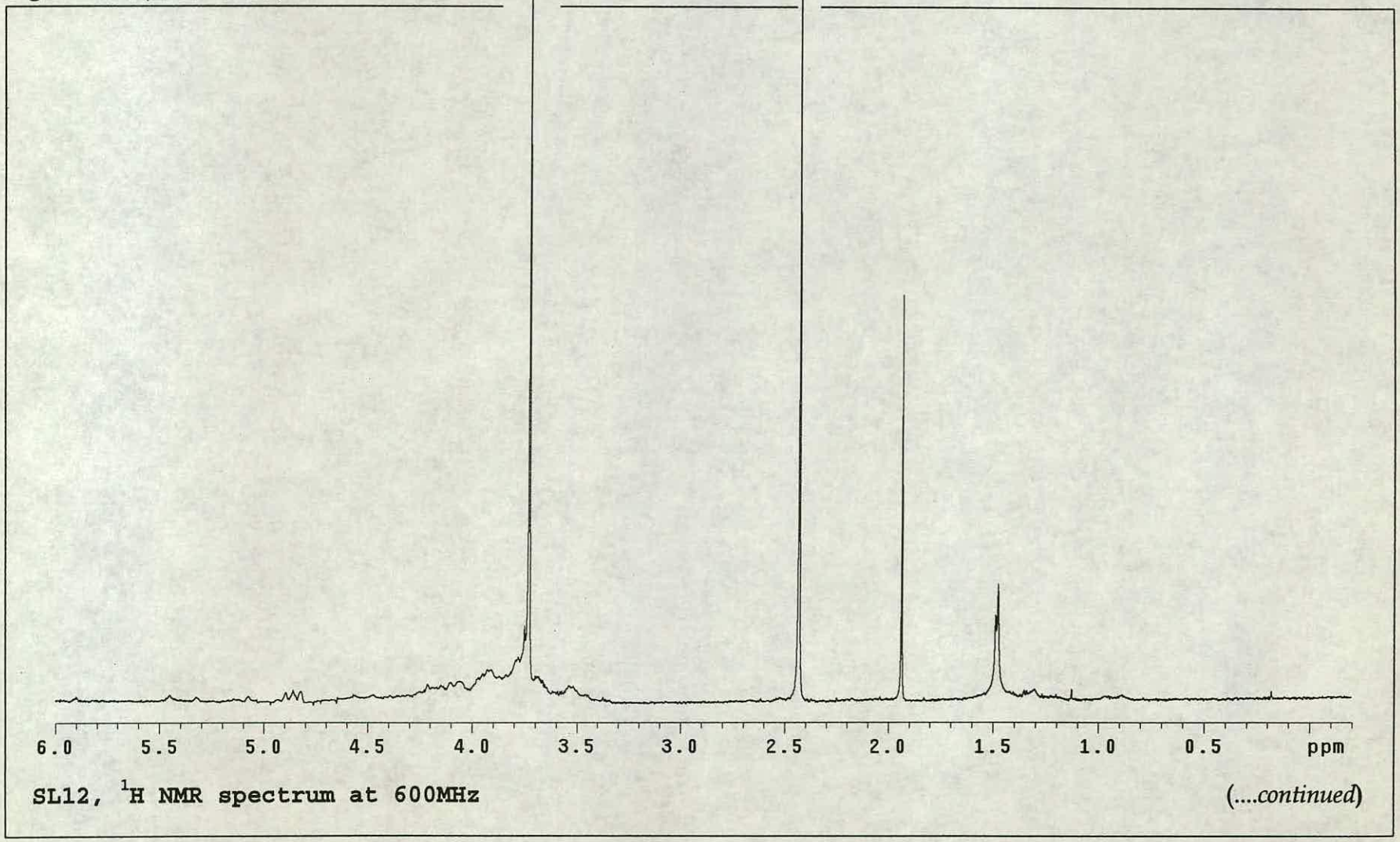

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Figure 3.15. (*...continued*)

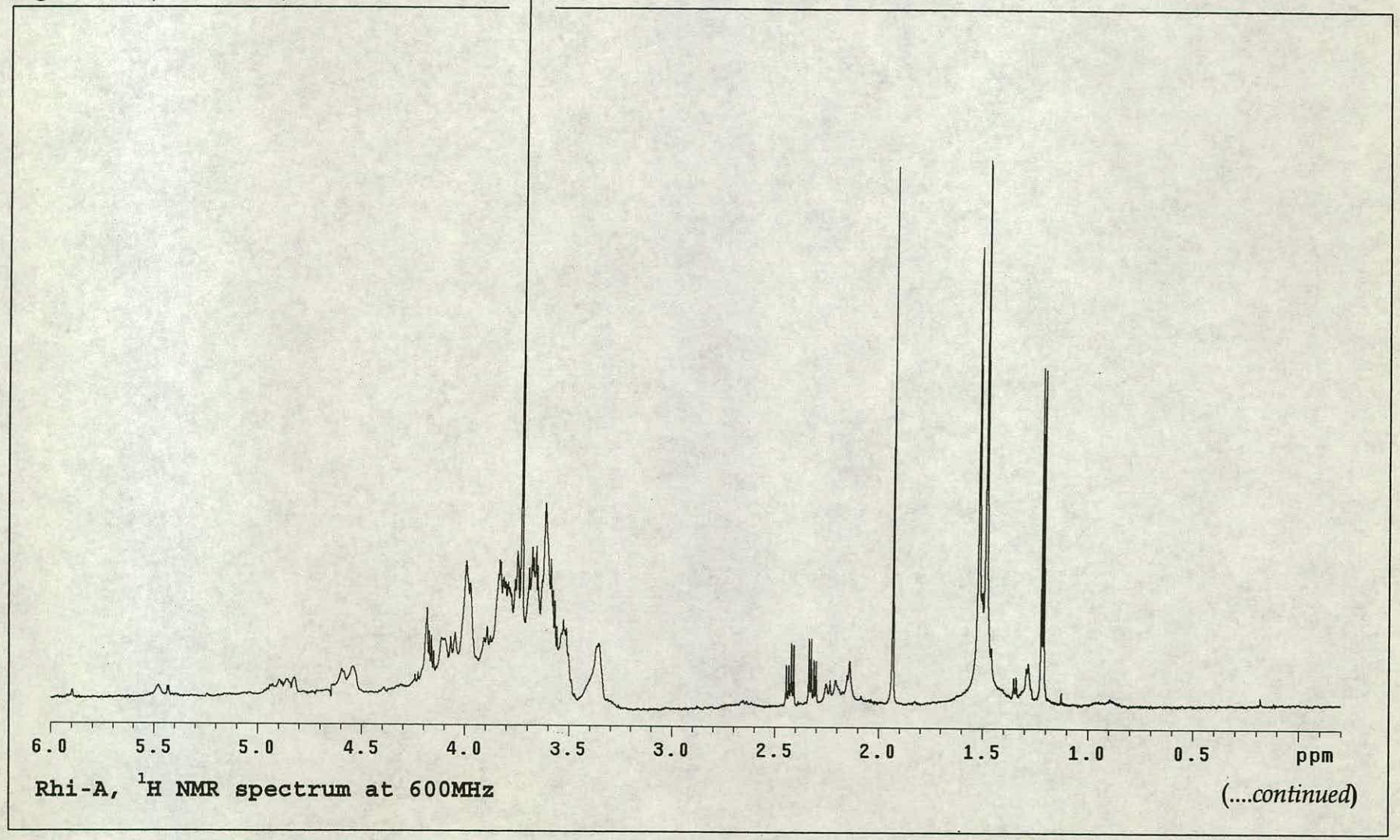

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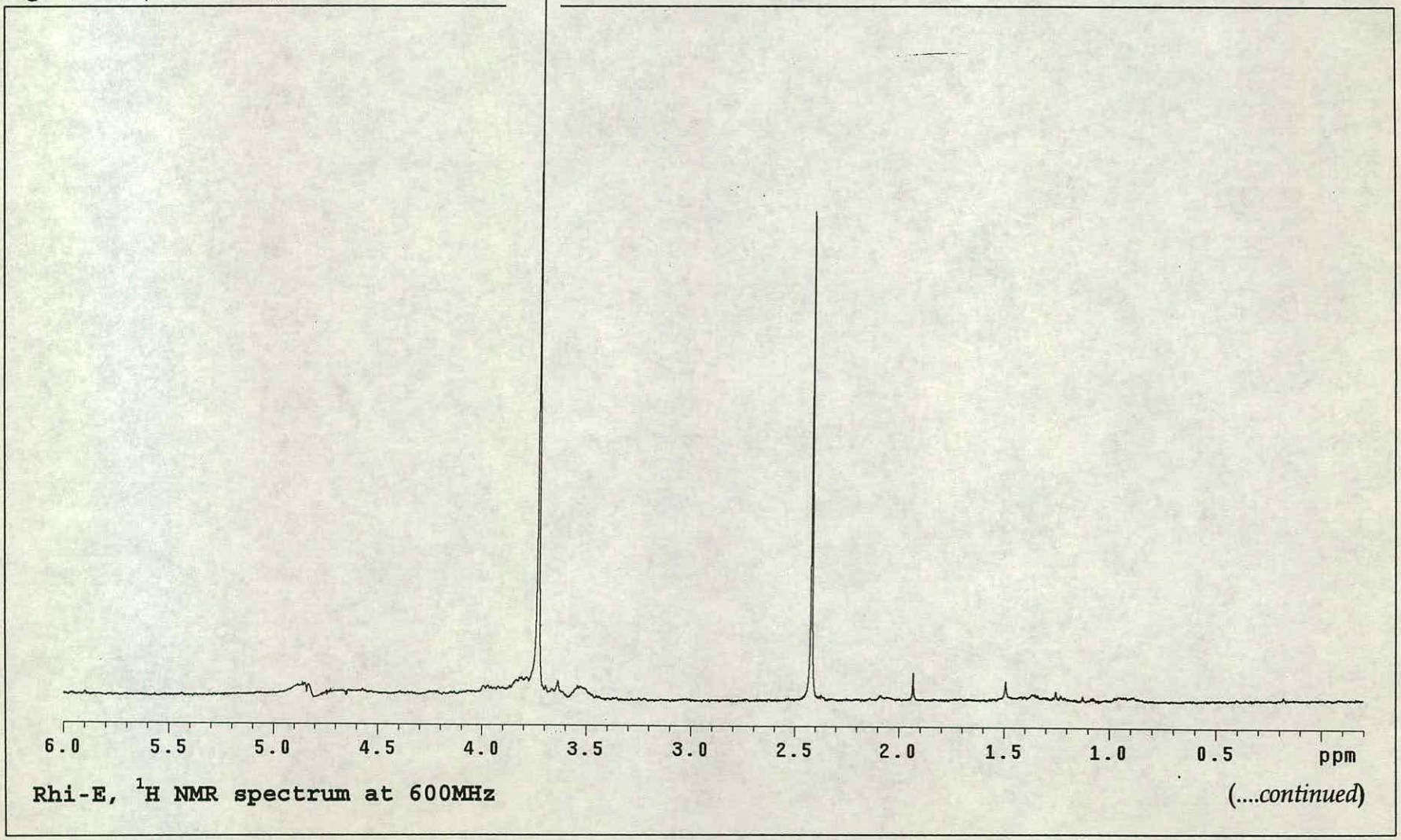
Figure 3.15. (*...continued*)



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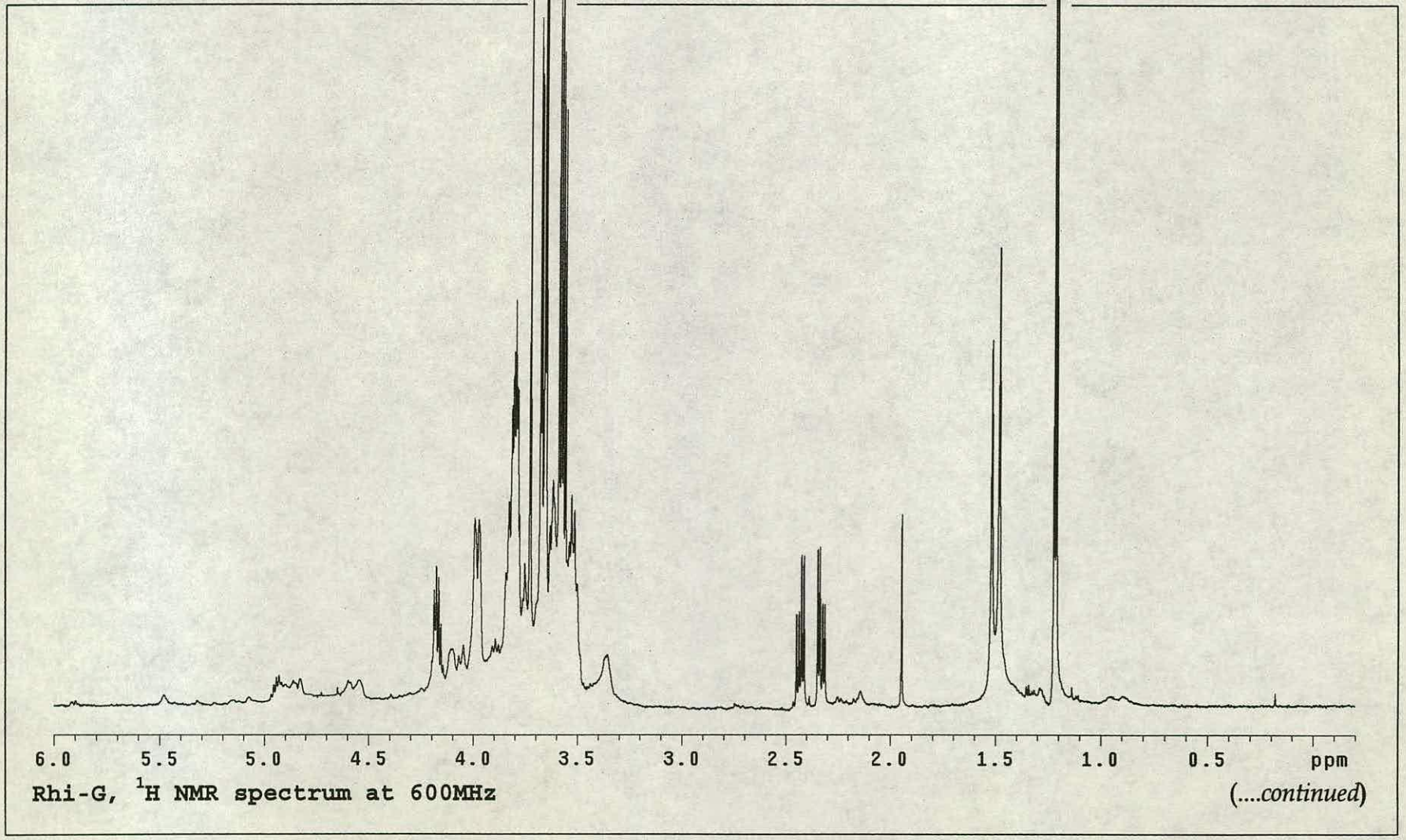

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Figure 3.15. (*...continued*)

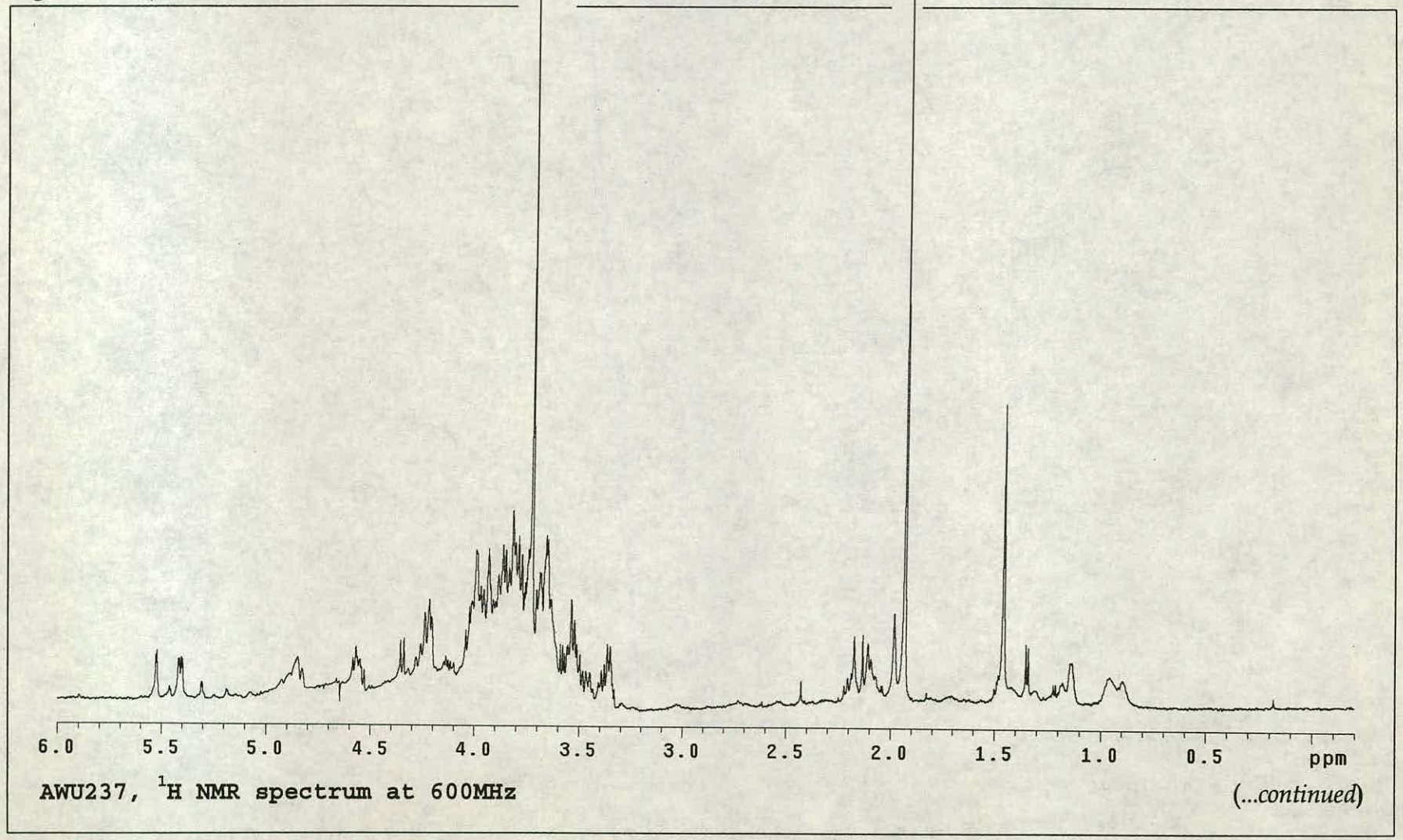

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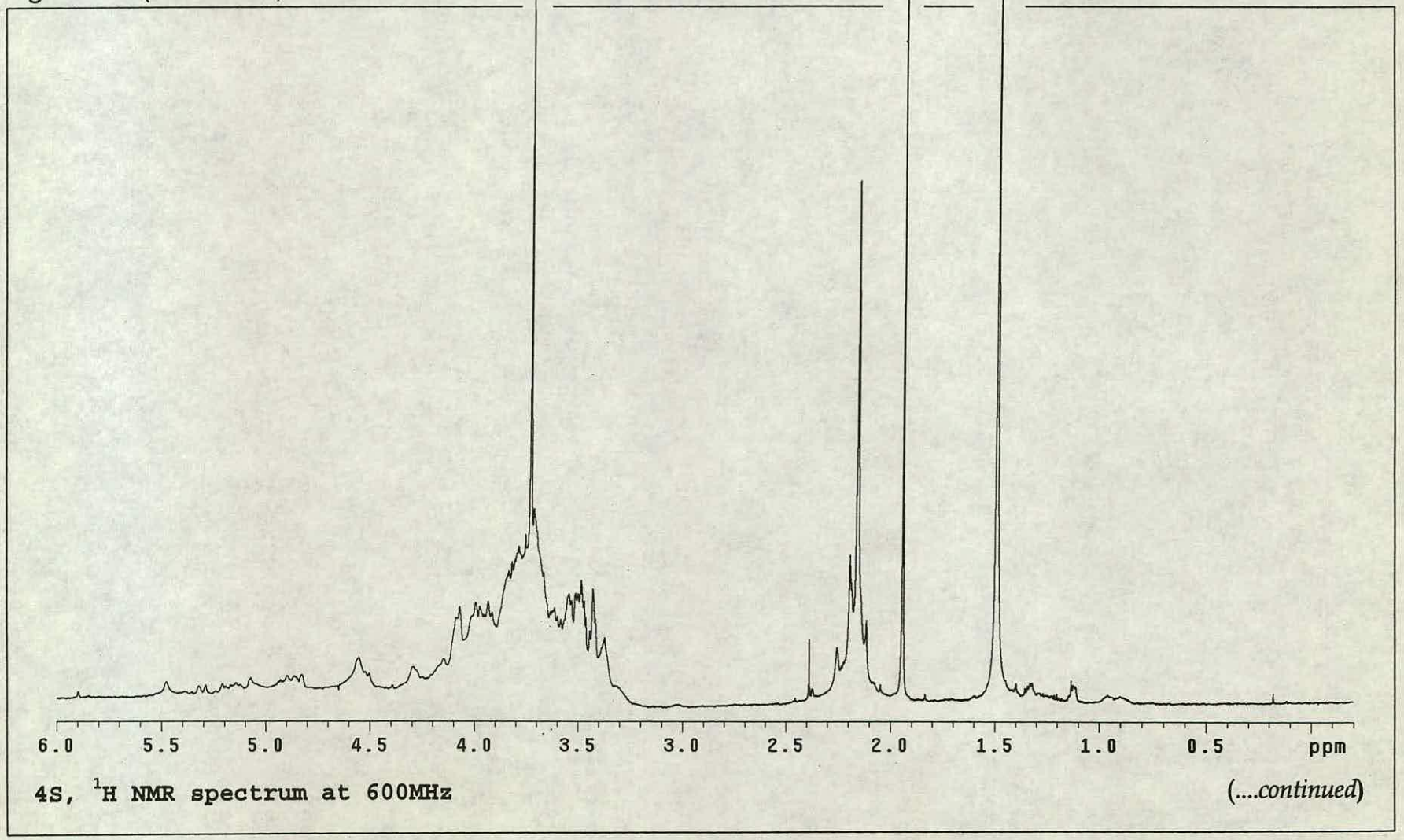
Figure 3.15. (*...continued*)



4S, ^{1}H NMR spectrum at 600MHz

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Figure 3.15. (*...continued*)

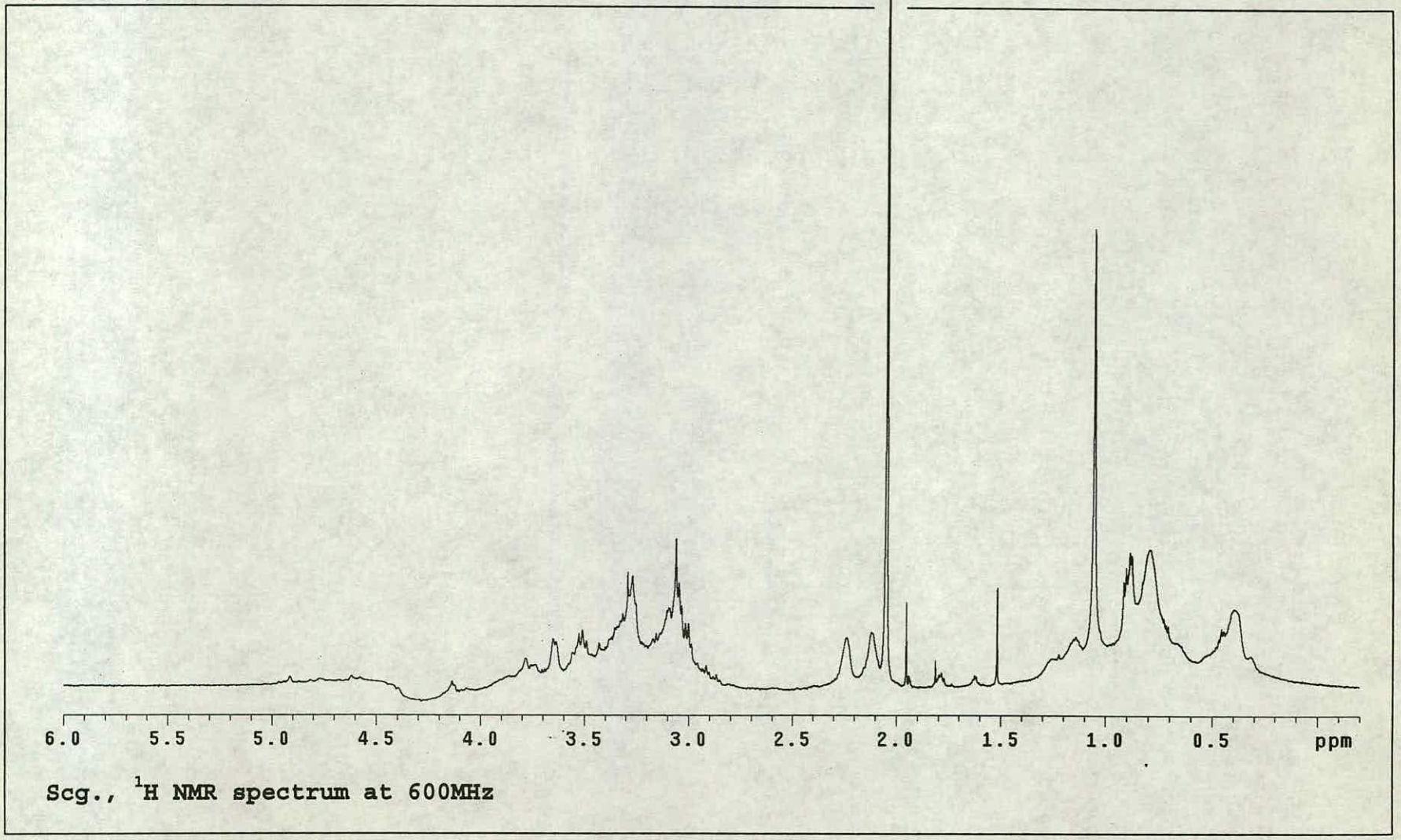


Table 3.9. Signals resonance in the typical range for chemical groups that contribute to the acylation of exopolysaccharides

Exopolysaccharide	Chemical shifts (ppm)		
	pyruvate	acetate	succinate
SL2a	1.2	1.91	2.2
SL2b	1.5**	1.95	2.45
SL3	1.45	1.95	2.45
SL4	1.5	1.95	2.45
SL5	1.5**	1.95	2.45**
SL7b	1.5**	1.95**	2.45
SL7c	1.5	1.95	-
SL9a	1.5**	1.95	2.45
SL9b	1.5**	1.95**	2.45
SL10	1.5	1.95*	2.45
SL11a	1.5**	1.95*	2.45
SL12	1.5	1.95	2.45
Rhi-A	1.5	1.95	-
Rhi-E	1.5*	1.95*	2.45
Rhi-G	1.5	1.95	2.45**
AWU237	1.45*	1.95	-
4S	1.5	1.95	2.15
Scg.	1.52*	1.95*	2.09

** small peaks*
***very small peaks (trace amounts)*

Although NMR is one of the best methods available to derive information on structure and composition of molecules, when it is used to analyse molecules such as exopolysaccharides in the present study, which are not 100% pure, it tends to read all the impurities thus adding some errors. These impurities can be caused by many sources including traces of other cell material. To avoid these errors, only significantly prominant information was taken into account. For instance, when the ratio between the peak areas for pyruvate, acetate and succinate groups was a figure like 0 : 20.5 : 1 respectively (Table 3.10, SL9a), the succinate signal was considered as negligible. The possible stoichiomeric ratio of pyruvate, acetate and succinate in a repeating unit of, for instance, exopolysaccharide of SL9a, was considered as 0 : 1 : 0 respectively. The ratios for all the exopolysaccharides tested are given in the table 3.10. Apart from SL7b, SL7c and SL9b the rest of the exopolysaccharides possessed at least one substituent group per repeating unit. When compared with most of known rhizobial exopolysaccharides, there were no significant differences in the contribution of these organic substituents to the structure of the exopolysaccharides analysed in the present study.

Table 3.10. The amounts of pyruvate, acetate and succinate in exopolysaccharides

Strain	Amounts[⊗] of			Ratio of peak areas given by the three groups	Possible stochiometric ratio in a repeating unit of EPS
	Pyruvate	Acetate	Succinate		
SL2a	10.3	2	5	5.1 : 1 : 2.5	2 : 0 : 1
SL2b	*	5.3	1	0 : 5.3 : 1	0 : 5 : 1
SL3	0.46	0.46	1.12	1 : 1 : 2.4	1 : 1 : 2
SL4	0.5	8.66	2.7	1 : 17 : 5.4	0 : 3 : 1
SL5	*	16.5	1.2	0 : 18 : 1	0 : 1 : 0
SL7b	*	*	-	0 : 0 : 0	0 : 0 : 0
SL7c	*	-	-	0 : 0 : 0	0 : 0 : 0
SL9a	*	36	1.75	0 : 20.5 : 1	0 : 1 : 0
SL9b	*	*	-	0 : 0 : 0	0 : 0 : 0
SL10	12.3	0.66	2.2	18 : 1 : 3.3	2 : 0 : 1
SL11a	0.33	1.21	9.85	1 : 3.6 : 29	0 : 0 : 1
SL12	4.33	2.83	6.75	1.5 : 1 : 2.4	3 : 2 : 5
Rhi-A	11.6	3.83	-	3 : 1 : 0	3 : 1 : 0
Rhi-E	0.1	0.1	4.1	1 : 1 : 41	0 : 0 : 1
Rhi-G	11.3	1.66	*	7 : 1 : 0	1 : 0 : 0
AWU237	6.6	12	-	1 : 2 : 0	1 : 2 : 0
4S	19.93	8.6	0	2 : 1 : 0	2 : 1 : 0
Scg.	15.44	1.13	15	14 : 1 : 13	1 : 0 : 1

* = *very small peaks*

[⊗] = *peak area / number of* ^{1}H *in the substituent group*

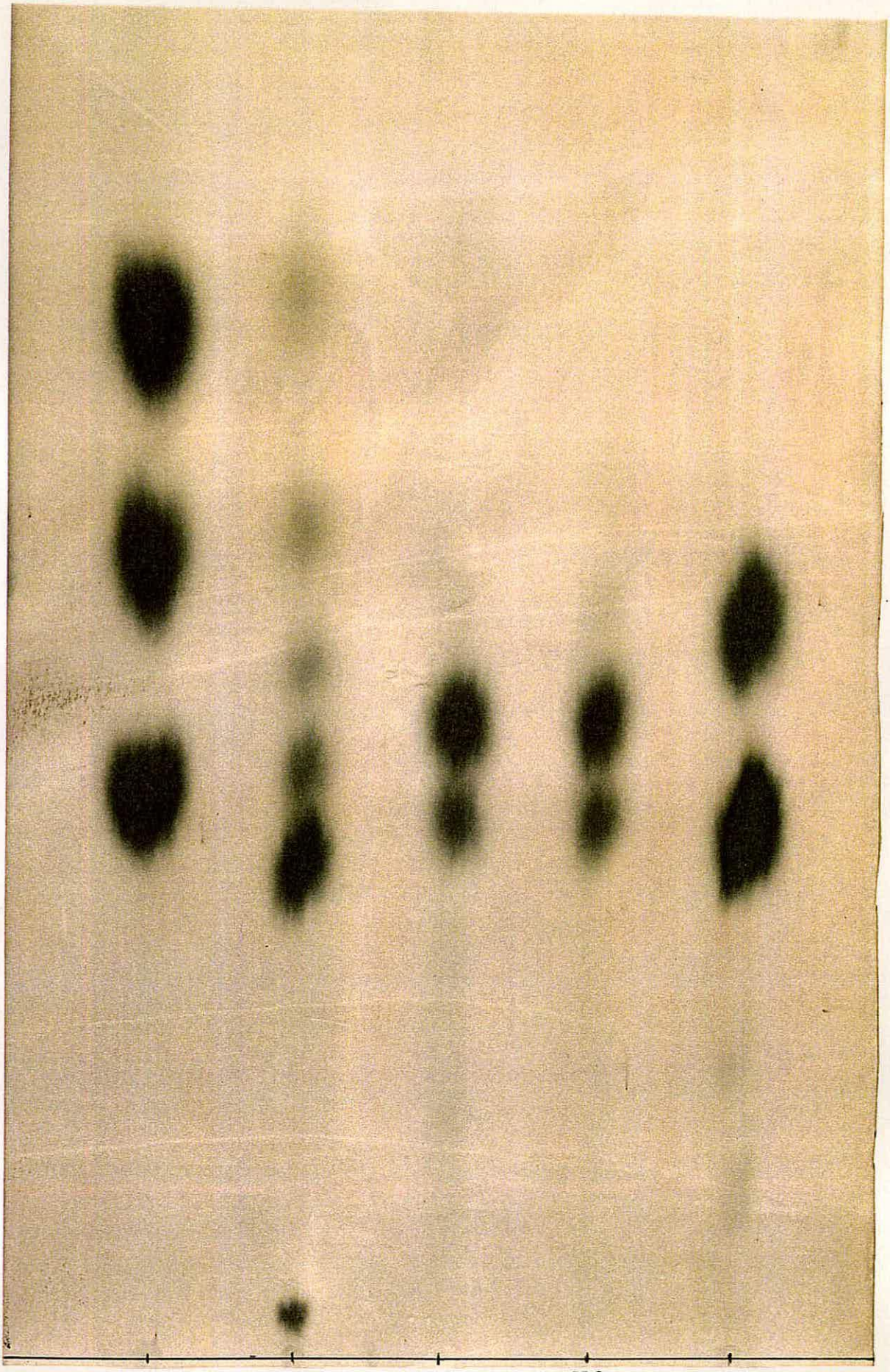
3.4.2. Analysis of the carbohydrate component

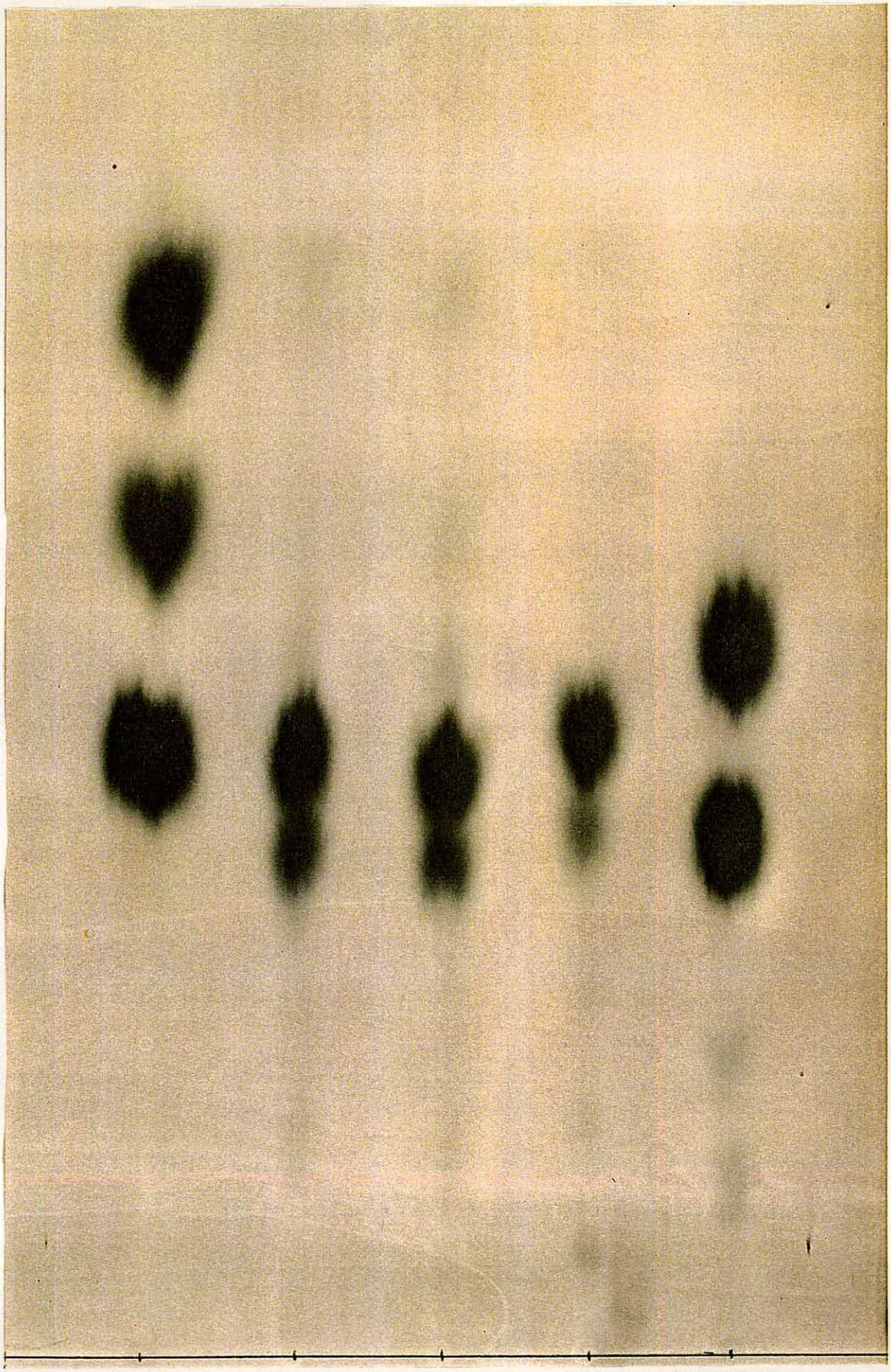
3.4.2.1. Analytical methods

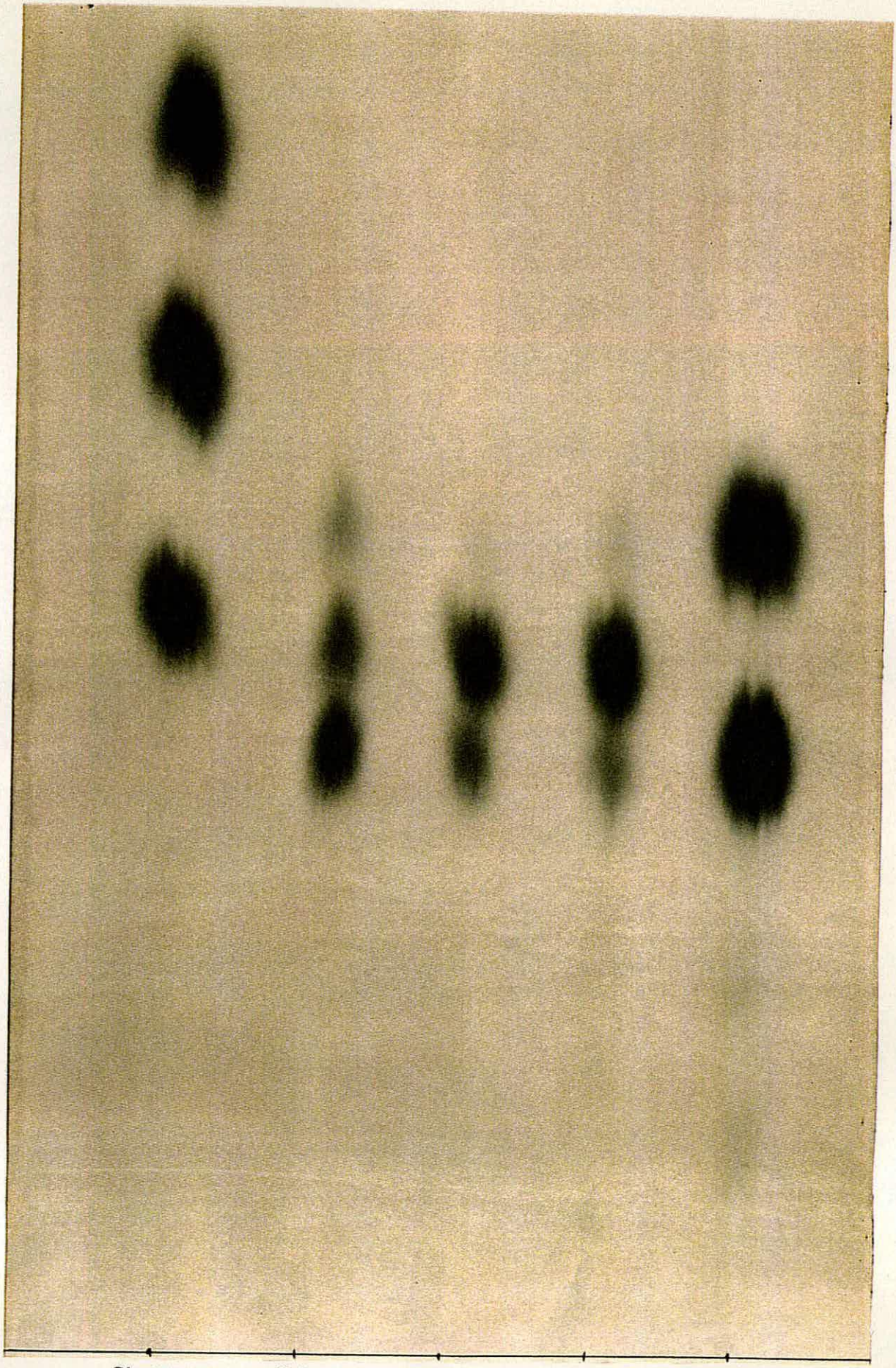
Paper chromatography

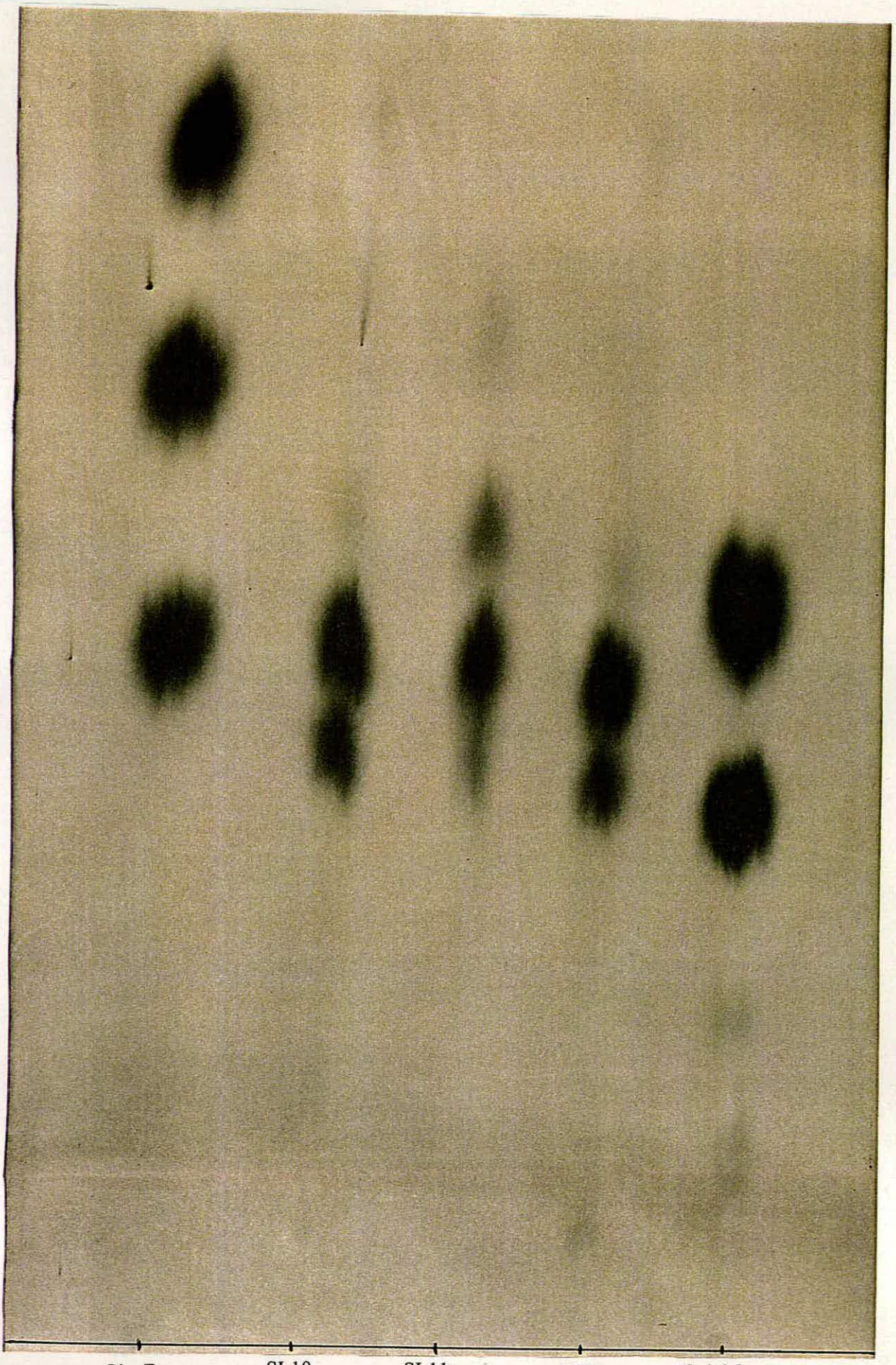
Acid hydrolysates of exopolysaccharides were analysed by paper chromatography which indicates the presence of each different constituent sugar of the polymers, and, if any, the sugar-uronic acids (aldobiouronic acids). Glucose, galactose, mannose, fucose and rhamnose, the most common neutral sugars present in rhizobial exopolysaccharides, were used as the reference standards in chromatograms and the results are given in figure 3.16 (pp. 175-181). Glucose and galactose were the predominant sugars in all the exopolysaccharides analysed. The presence of mannose was also detected in many of them, though mostly as traces. However, SL11a exopolysaccharide had a significant amount of mannose. It also had traces of fucose while SL5 and Rhi-A possessed rhamnose in trace amounts. The presence of rhamnose in H_3 and 4S was significant. SL2a composition was remarkably different from other exopolysaccharides by having all the above mentioned sugars, i.e., rhamnose, fucose, mannose, glucose and galactose present. The sizes and intensities of the spots on chromatograms given by each sugar were compared to obtain a preliminary understanding about the relative ratio of sugars (table 3.11). It could be concluded that almost all the exopolysaccharides had a higher glucose:galactose ratio, ranging from about

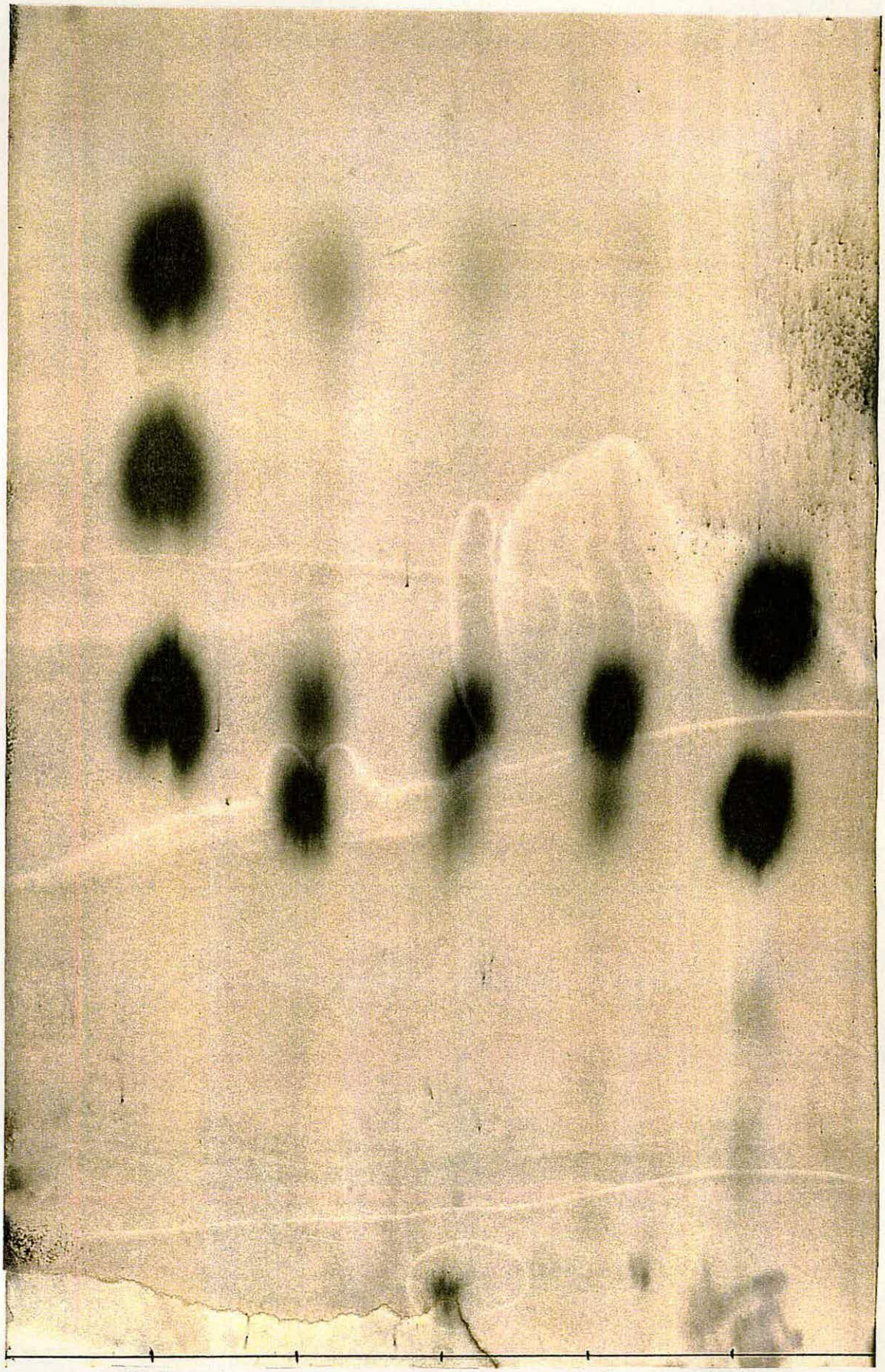
Figure 3.16. Paper chromatograms of exopolysaccharides (*overleaf*)
(standards were on both sides of the paper; papers were cut into two after developed. Distance travelled: glc< fuc< rha; gal<man)

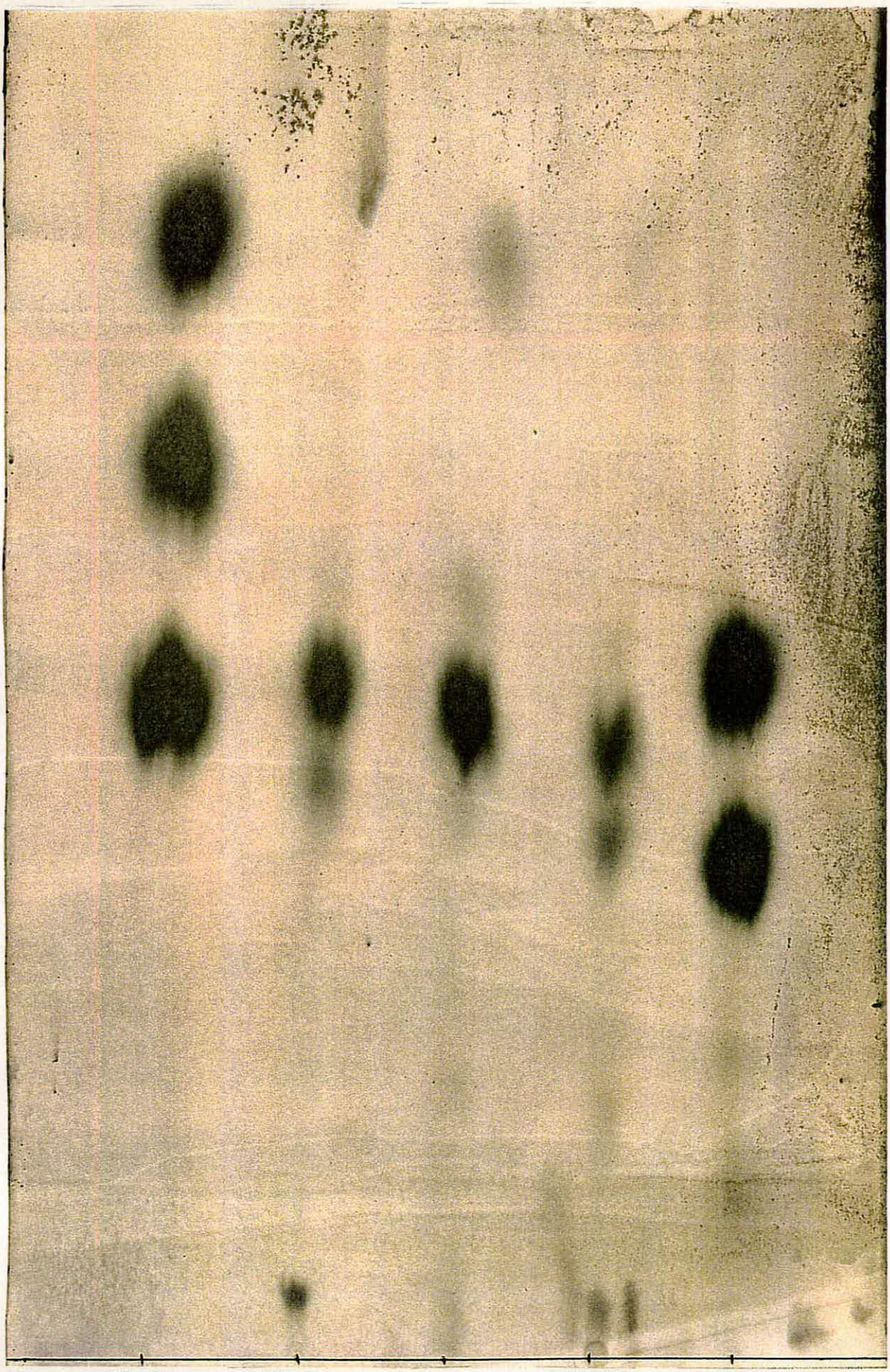

Glc, Fuc
Rha
SL2a
SL2b
SL3
Gal, Man

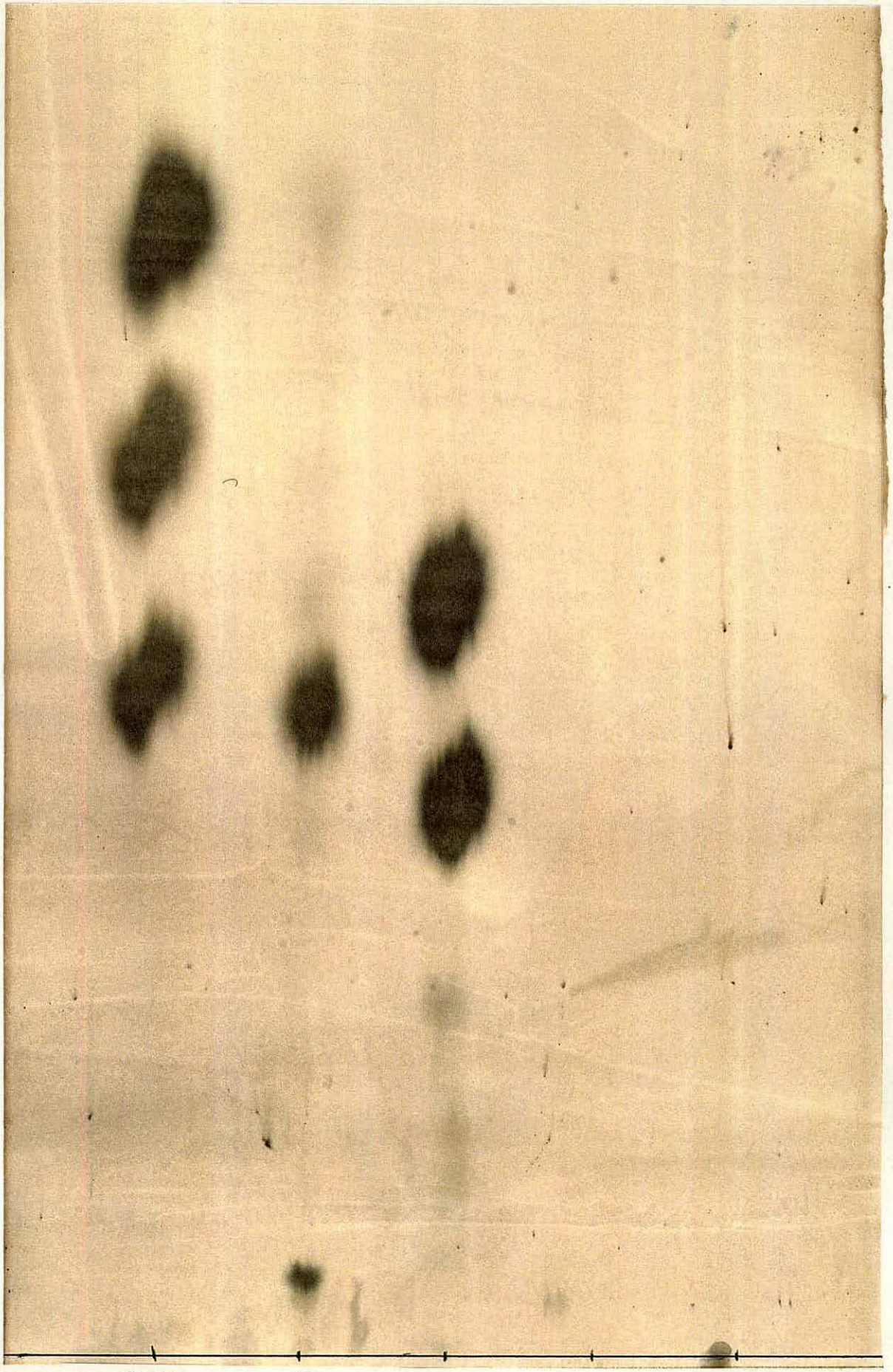

Glc, Fuc
Rha
SL4
SL5
SL7b
Gal, Man


Glc, Fuc
Rha
SL7c
SL9a
SL9b
Gal, Man


Glc, Fuc
Rha
SL10
SL11a
SL12
Gal, Man


Glc, Fuc
Rha
SL12
Rhi-A
Rhi-E
Gal, Man


Glc, Fuc
Rha
Rhi-G
H3
AWU237
Gal, Man


Glc, Fuc
Rha
4S
Gal, Man

5:3.upto 4:1. H_3 had only traces of galactose when compared to glucose. SL2a was again exceptional by having more galactose than glucose, i.e., about twice as much as glucose. SL7c also had more galactose than glucose but the difference was only slight.

Table 3.11. Relative amounts of neutral sugars present in exopolysaccharides: a rough estimation by paper chromatography

Exopolysaccharide	Rha.	Fuc.	Man.	Glc.	Gal.
SL2a	1	1	1	2	4
SL2b	-	-	-	5	3
SL3	-	-	tr.	5	3
SL4	-	-	-	4	2
SL5	tr.	-	-	4	2
SL7b	-	-	-	4	1
SL7c	-	-	1	3	4
SL9a	-	-	-	4-5	2
SL9b	-	-	tr.	4	2
SL10	-	-	tr.	4-5	2
SL11a	-	tr	2	4	1
SL12	-	-	tr.	4	2
Rhi-A	tr.	-	-	4	1
Rhi-E	-	-	-	4	1
Rhi-G	-	-	-	4	1
AWU237	-	-	-	4	2
4S	-	-	tr.	4-5	tr.
*H_2	1	-	-	3	1
H_3	1	-	1	4-5	tr.
*USDA110	-	1	1	4	1
*11-160-M_7					
Scg.	-	-	-	5	2

**except these, the other exopolysaccharides were purified prior to hydrolysis* (p. 93)

Rha.= Rhamnose, Fuc.= Fucose, Man.= Mannose
Glc.= Glucose, Gal.= Galactose, Scg.= Succinoglycan
tr.= trace amounts

Detection of uronic acid component by paper chromatography

Sugar-uronic acids (aldobiouronic acids) such as glucuronic acid and galacturonic acid occur as constituent parts of the backbone of some rhizobial exopolysaccharides. When compared to neutral sugars, these compounds travel only short distances on paper choromatograms run with organic solvents. The presence of uronic acids was detected on several exopolysaccharides analysed by paper chromatography, i.e., SL2a, Rhi-A, Rhi-G, AWU237 and 4S (Figure 3.16). Some uronic acid was probably lost during the neutralization process through adsorption to the ion-exchange resins in the process of neutralising the acid hydrolysates.

Chemical determination of uronic acid component

The uronic acid content as a %($^{w}/_{w}$) of exopolysaccharide was analysed chemically by Blumenkrantz and Asboe-Hansen (1973) method (p. 102). Table 3.12 shows the results which give the total weight of different uronic acids occur in exopolysaccharide structure. SL2b, SL4, SL5 and SL9b had only insignificant amounts of sugar-uronic acids while, in contrast, about 50%($^{w}/_{w}$) of WGR234, AWU237 and 4S exopolysaccharides were composed of them. SL2a and SL3 had about 30%($^{w}/_{w}$) sugar-uronic acids and the rest of the exopolysaccharides studied showed a uronic acid component ranging between about 10%-20%($^{w}/_{w}$).

Table 3.12. Uronic acid content of rhizobial exopolysaccharides

Rhizobial strains	$\%^{w}/_{w}$ uronic acids in exopolysaccharides
SL2a	31.18
SL2b	3.01
SL3	34.48
SL4	3.37
SL5	1.74
SL7b	17.41
SL7c	22.24
SL9a	13.95
SL9b	5.27
SL10	12.55
SL11a	16.37
SL12	16.68
WGR234	50.57
AWU237	55.06
4S	54.62

High performance liquid chromatography

The results of the HPLC analysis of exopolysaccharides, using Dionex equipment are given in figure 3.17. Fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid were the standards used in the analysis. Table 3.13 (p.204) gives the summary of the HPLC results. Majority of the exopolysaccharides were basically similar to known rhizobial exopolysaccharides. However, the presence of rhamnose as trace amounts is unusual. SL2a and SL7c are significantly different from other exopolysaccharides by having more galactose than glucose.

Figure 3.17. HPLC analysis of exopolysaccharides *(overleaf)*
(Elusion times in minutes: rha=5.9, fuc=9.6, man=14.02, glc=16.2, gal=20.3, galA=48.7, glcA=60.01)

Figure 3.17. HPLC analysis of exopolysaccharides

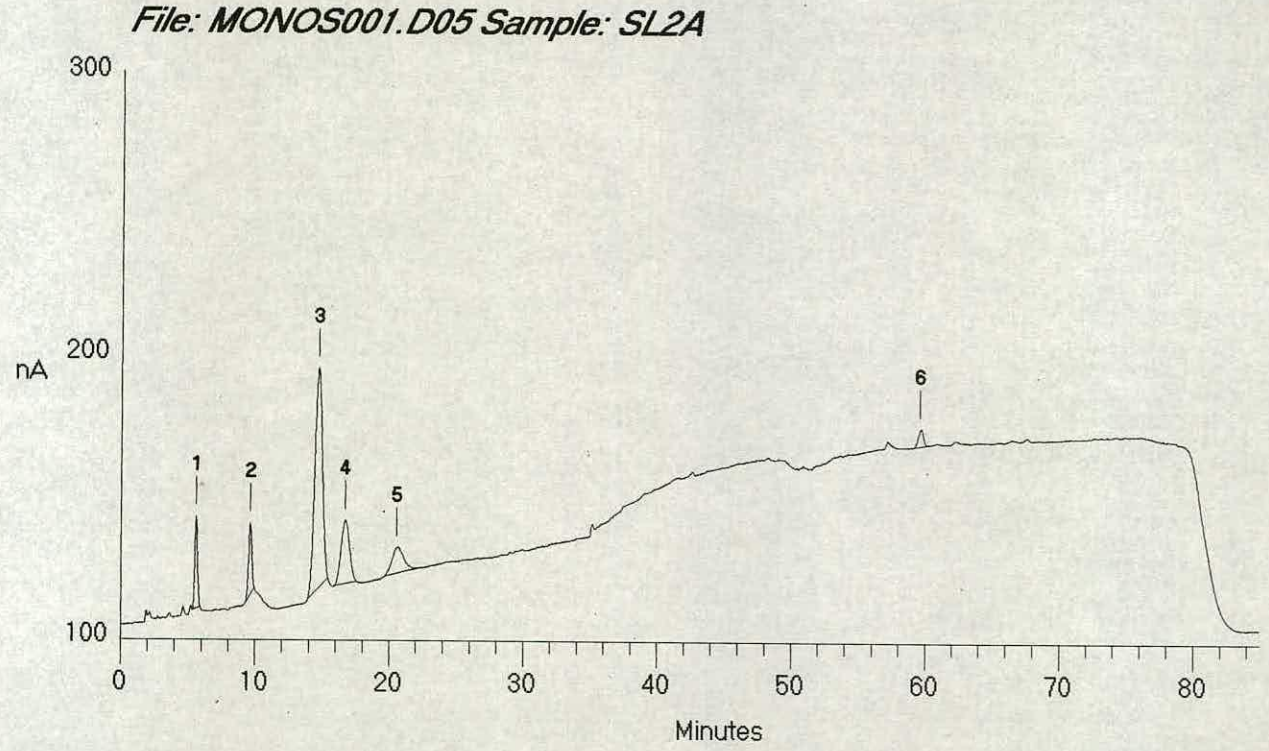


Figure 3.17. (continued)

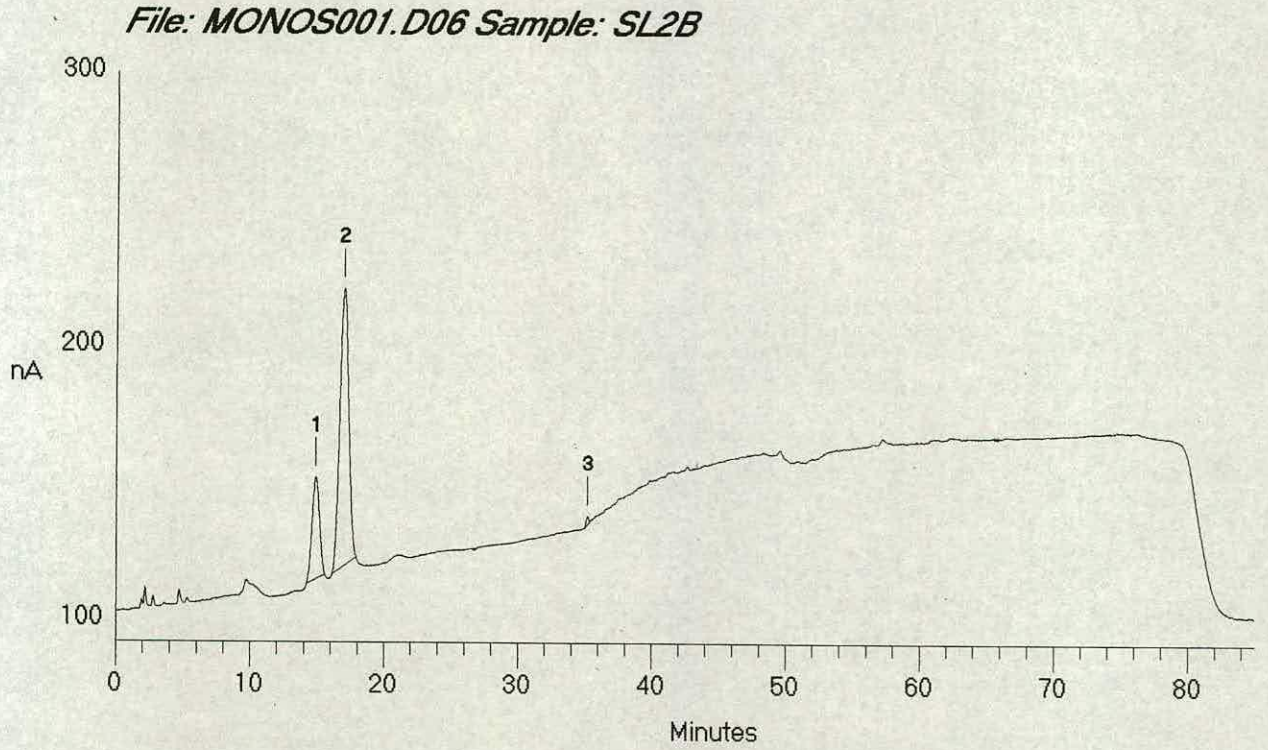


Figure 3.17. (continued)

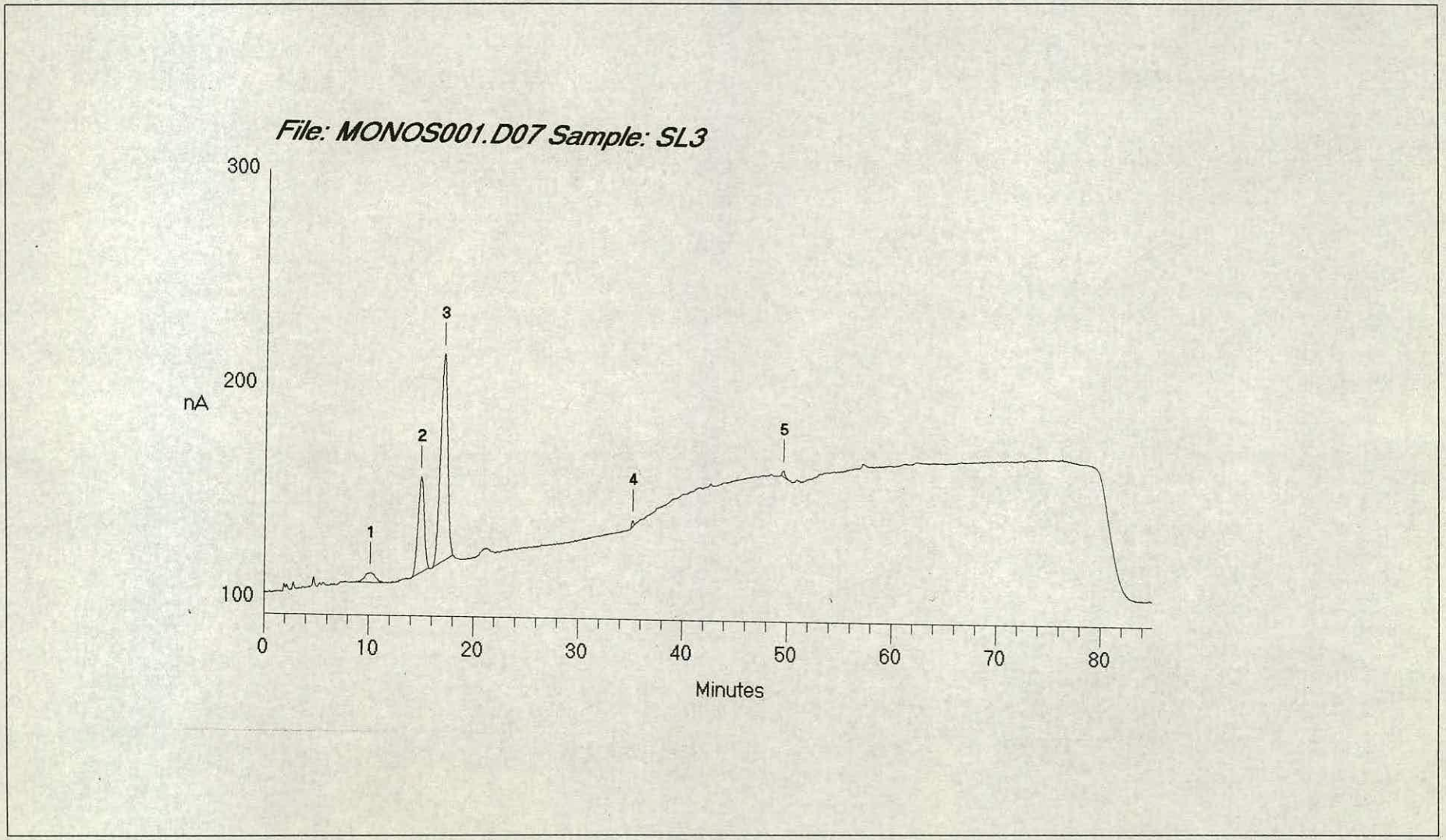


Figure 3.17. (continued)

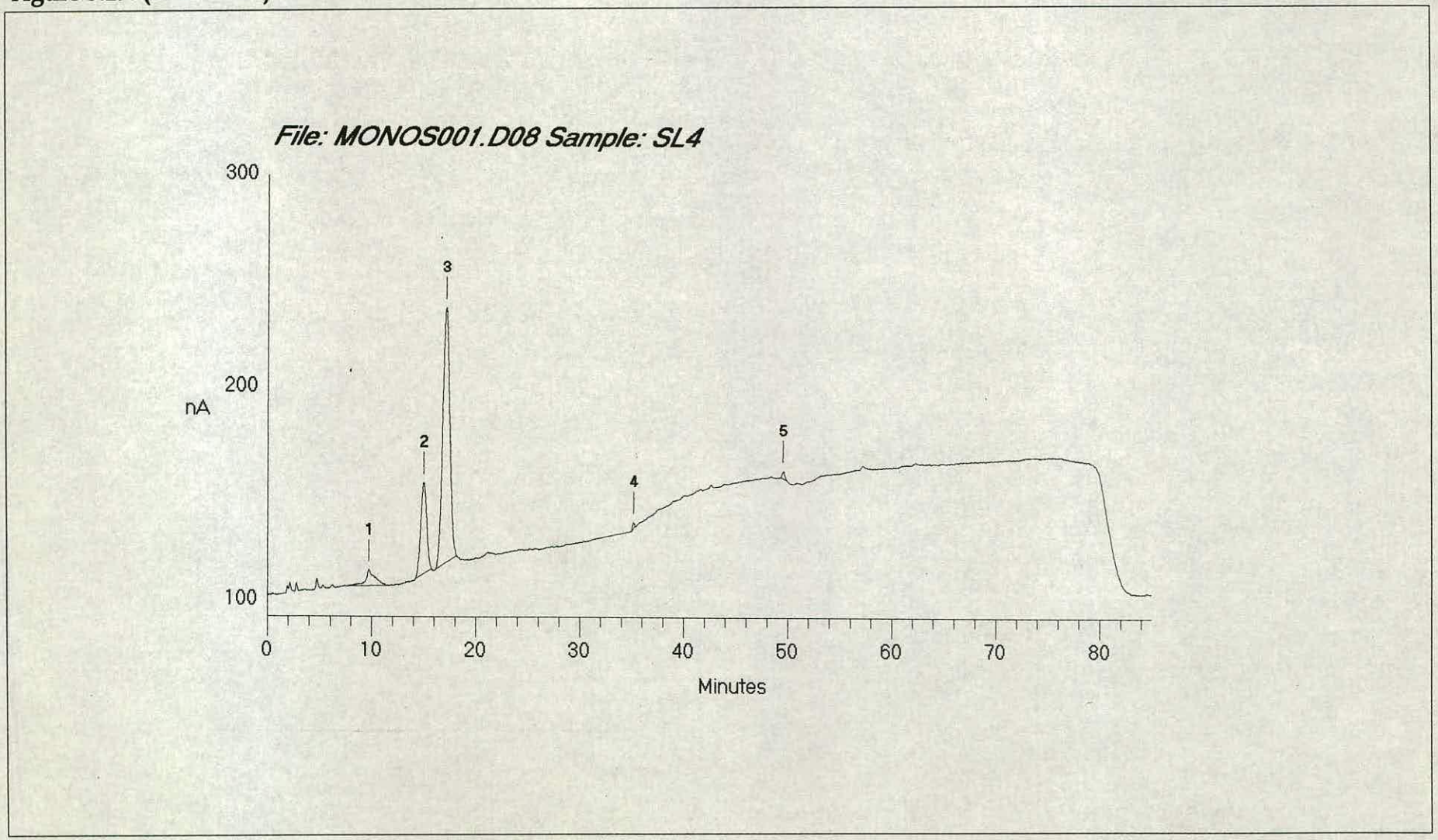


Figure 3.17. (continued)

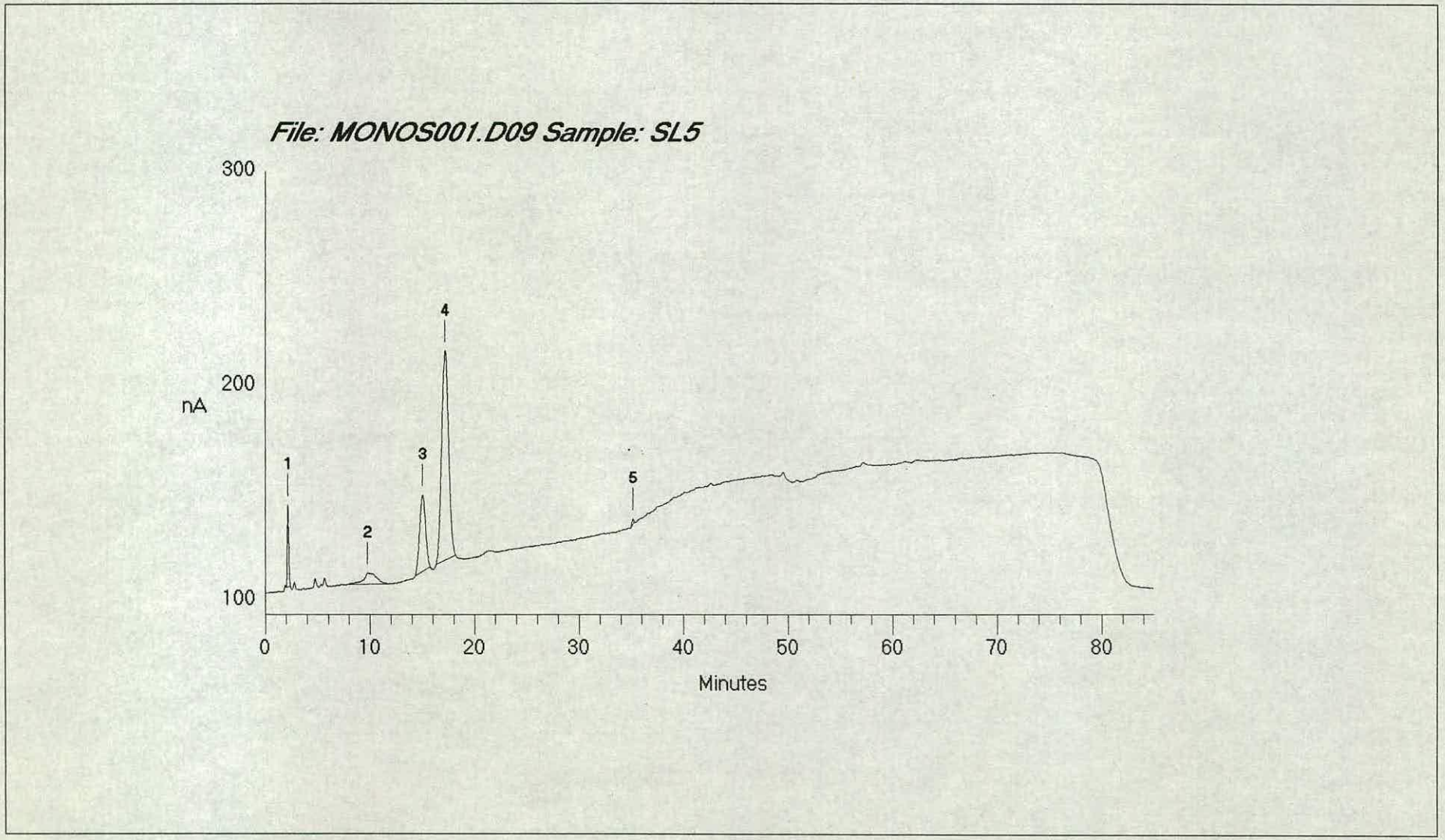


Figure 3.17. (continued)

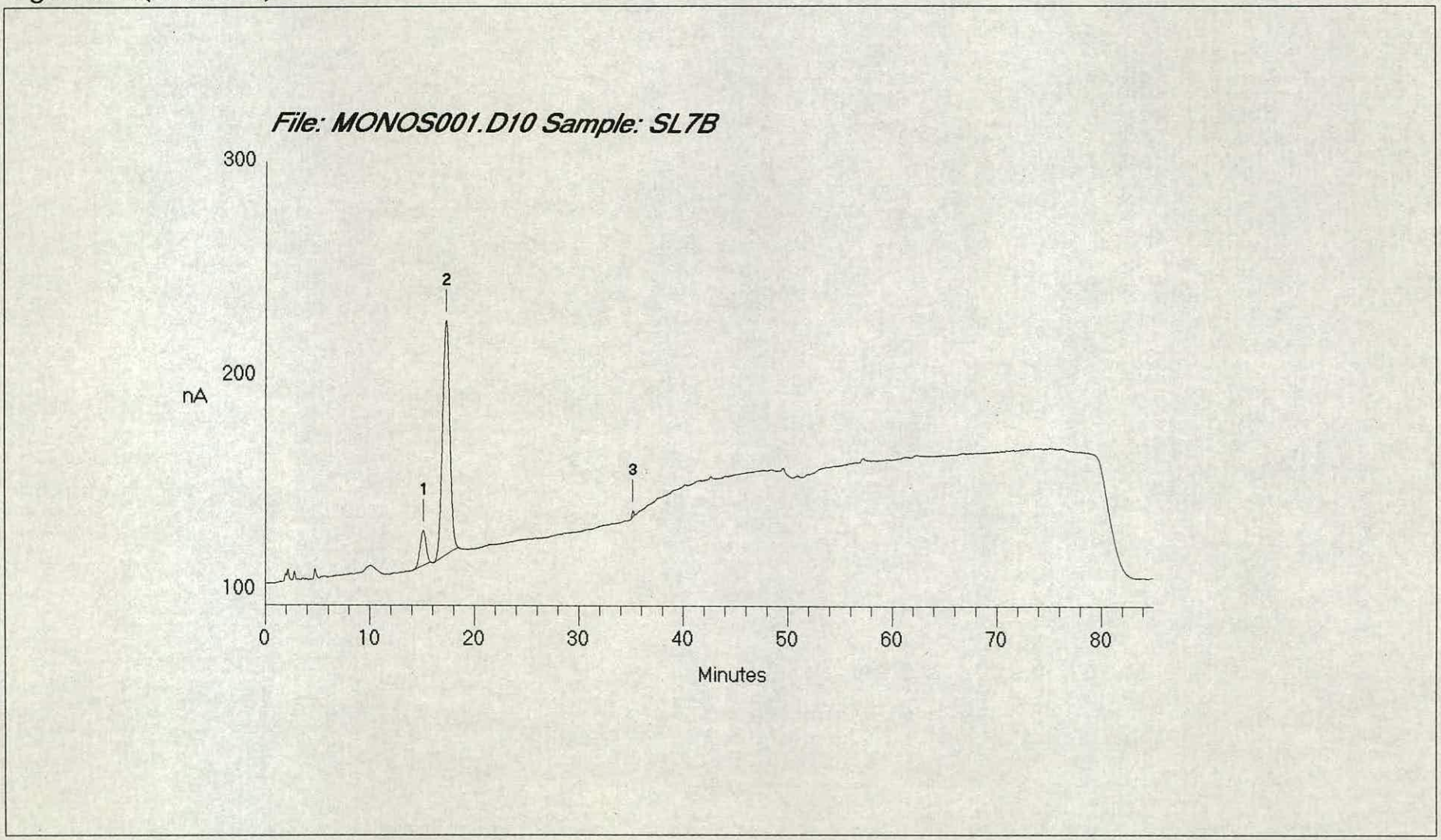


Figure 3.17. (continued)

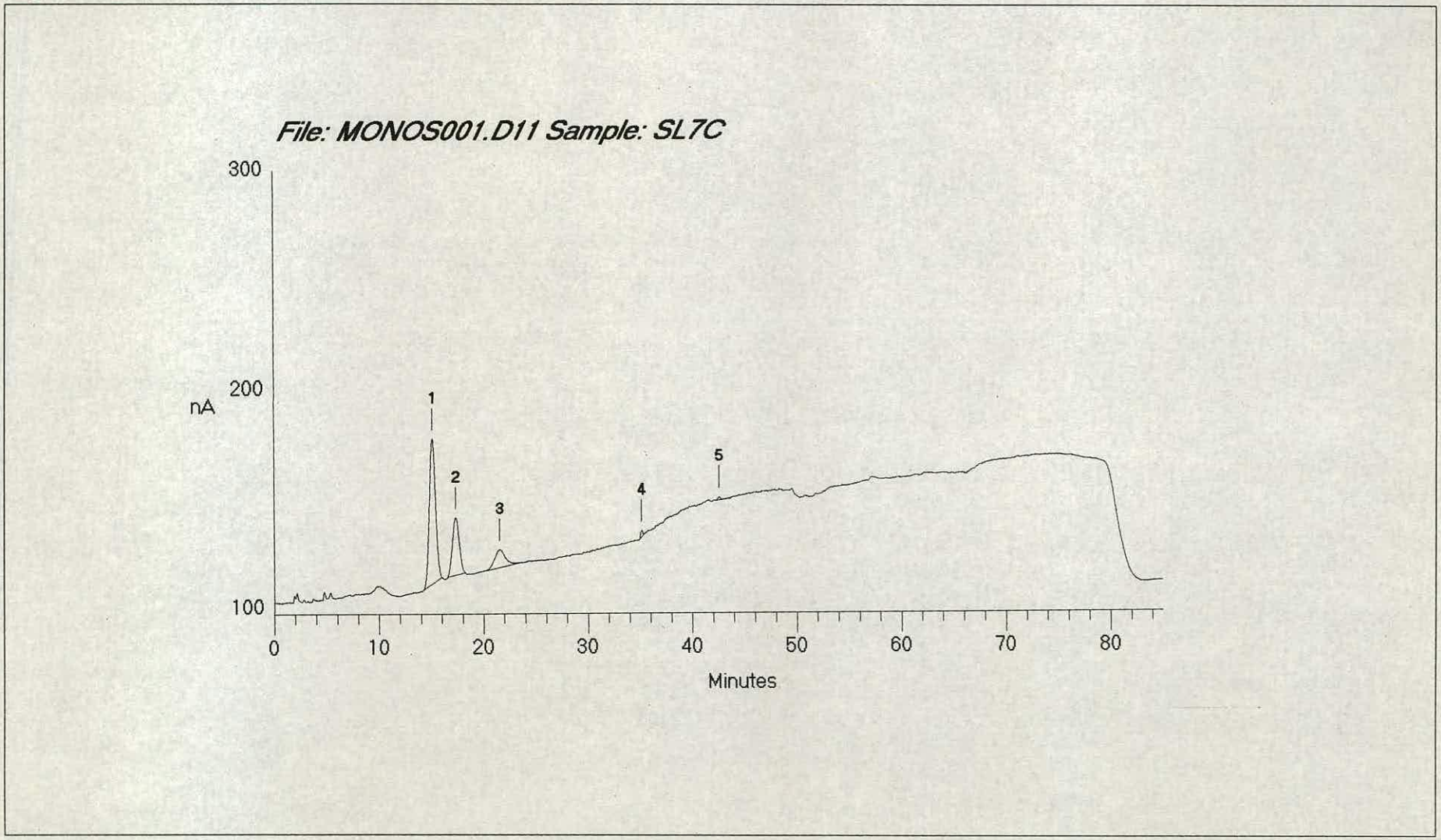


Figure 3.17. (continued)

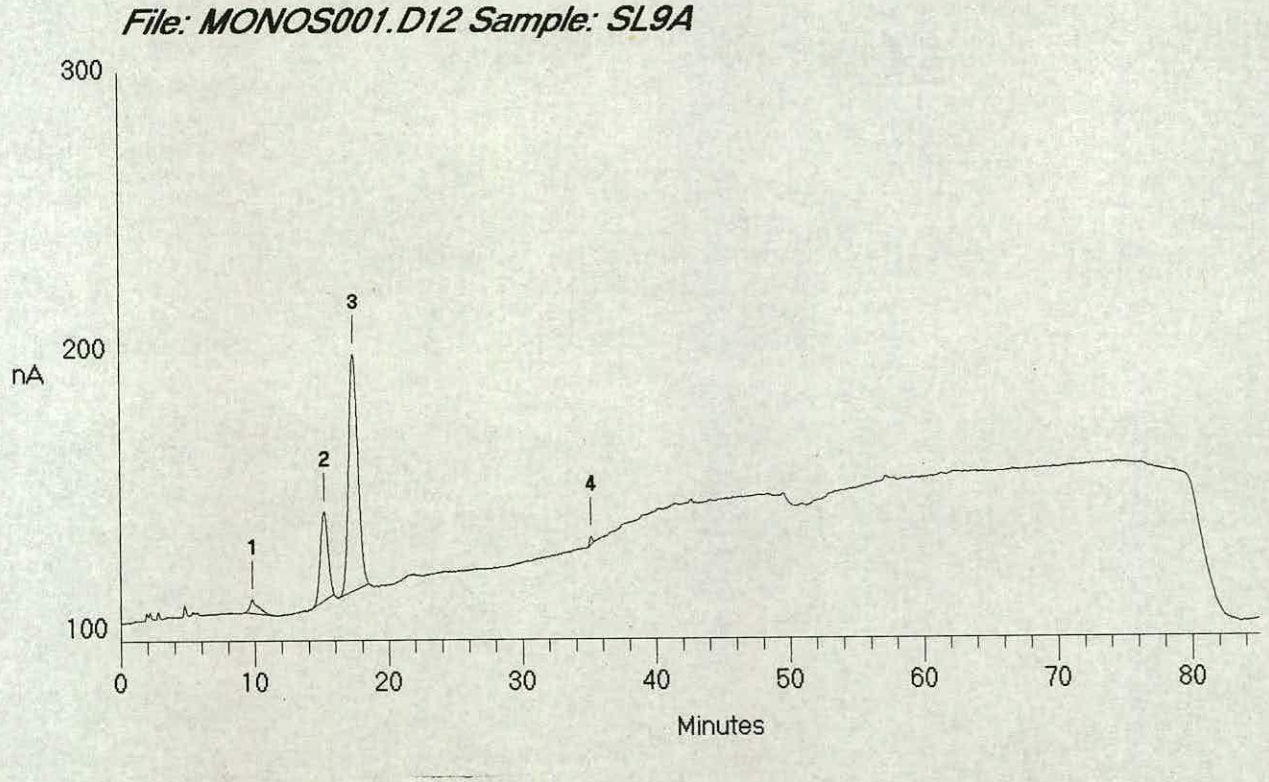


Figure 3.17. (continued)

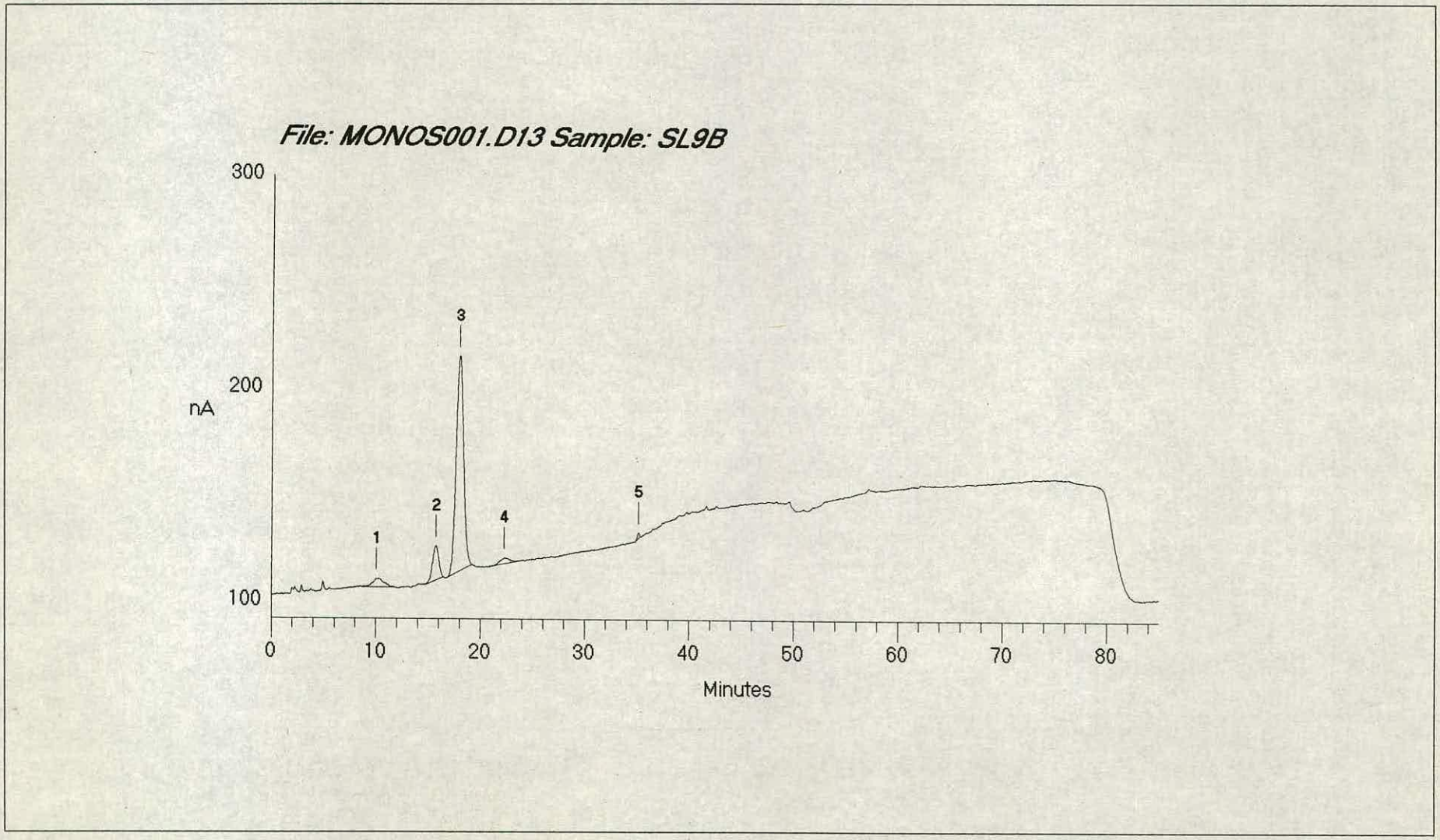


Figure 3.17. (continued)

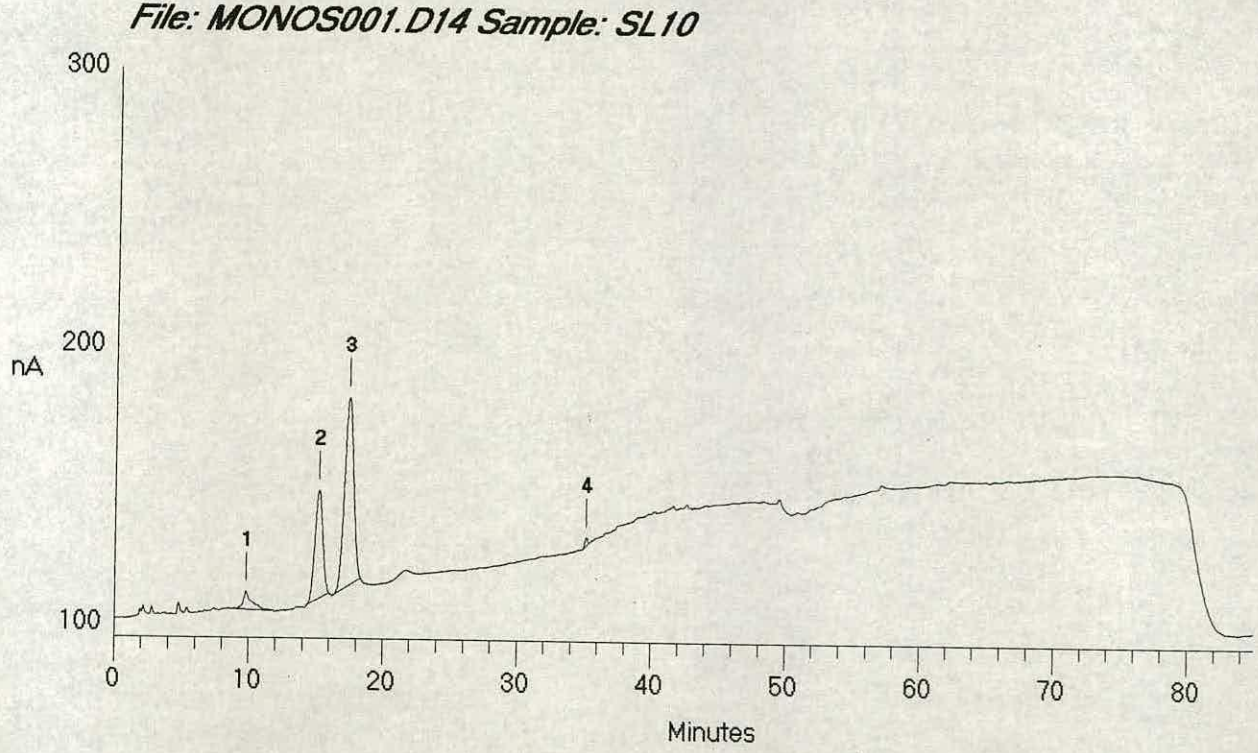


Figure 3.17. (continued)

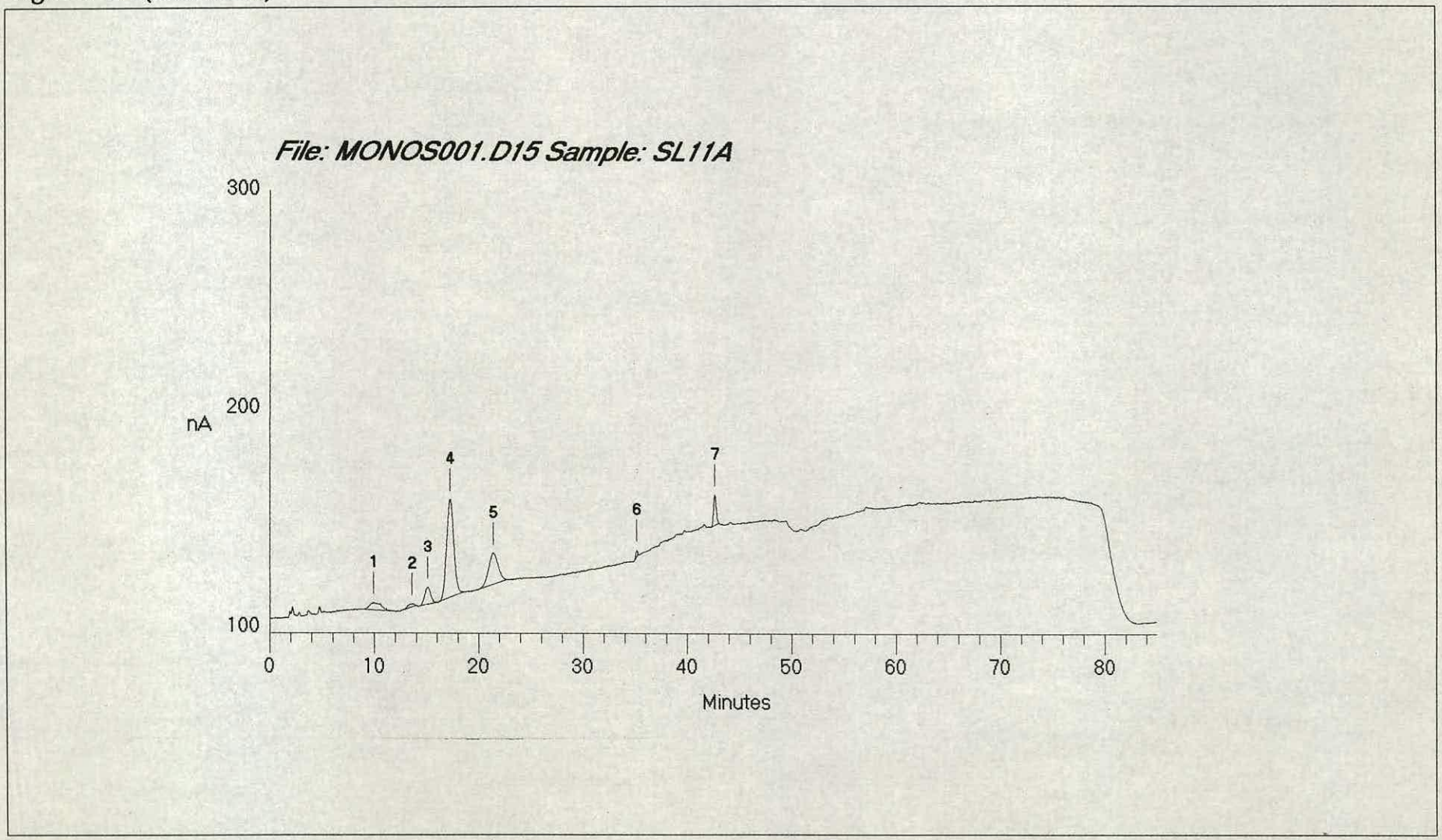


Figure 3.17. (continued)

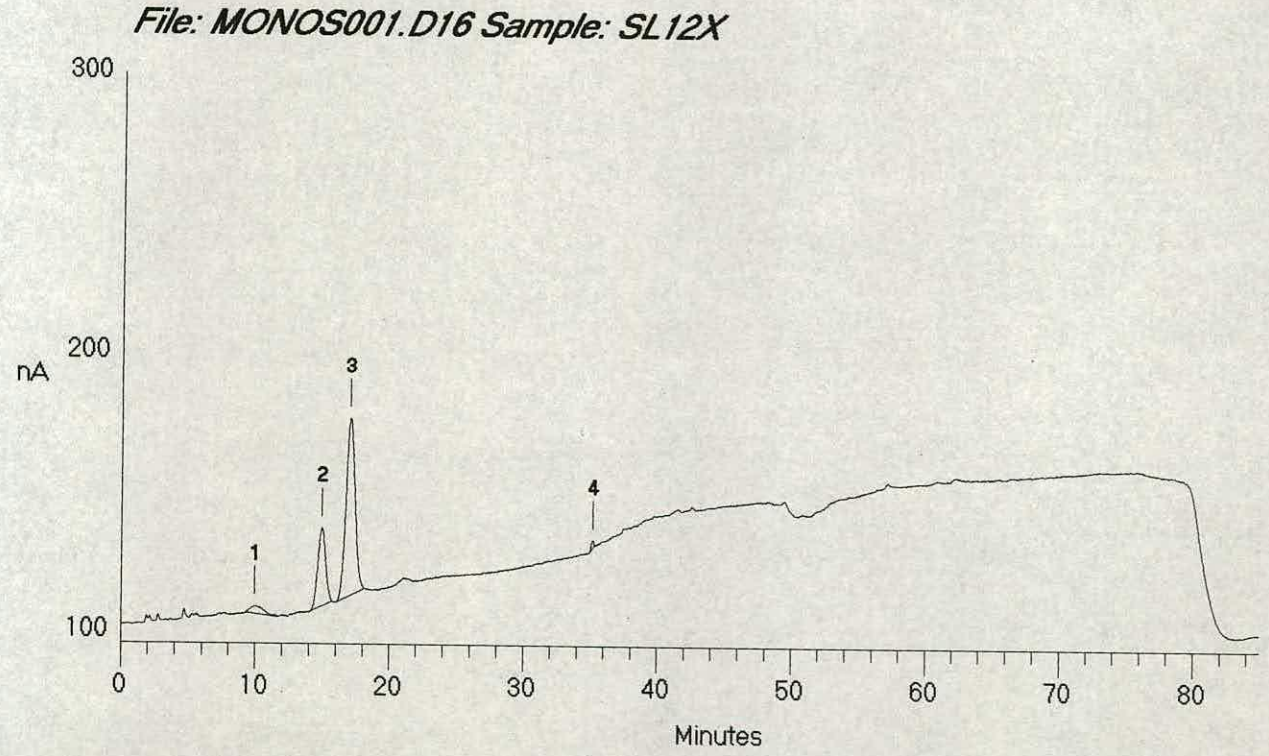
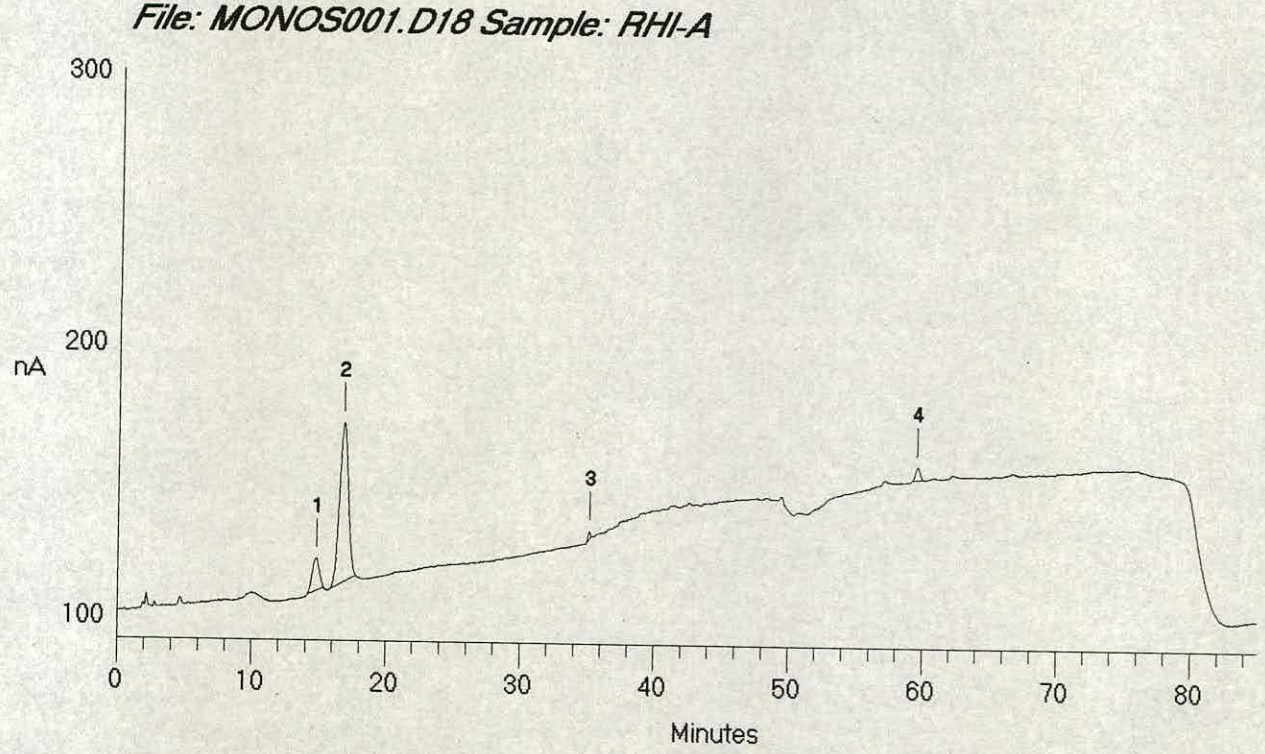


Figure 3.17. (continued)



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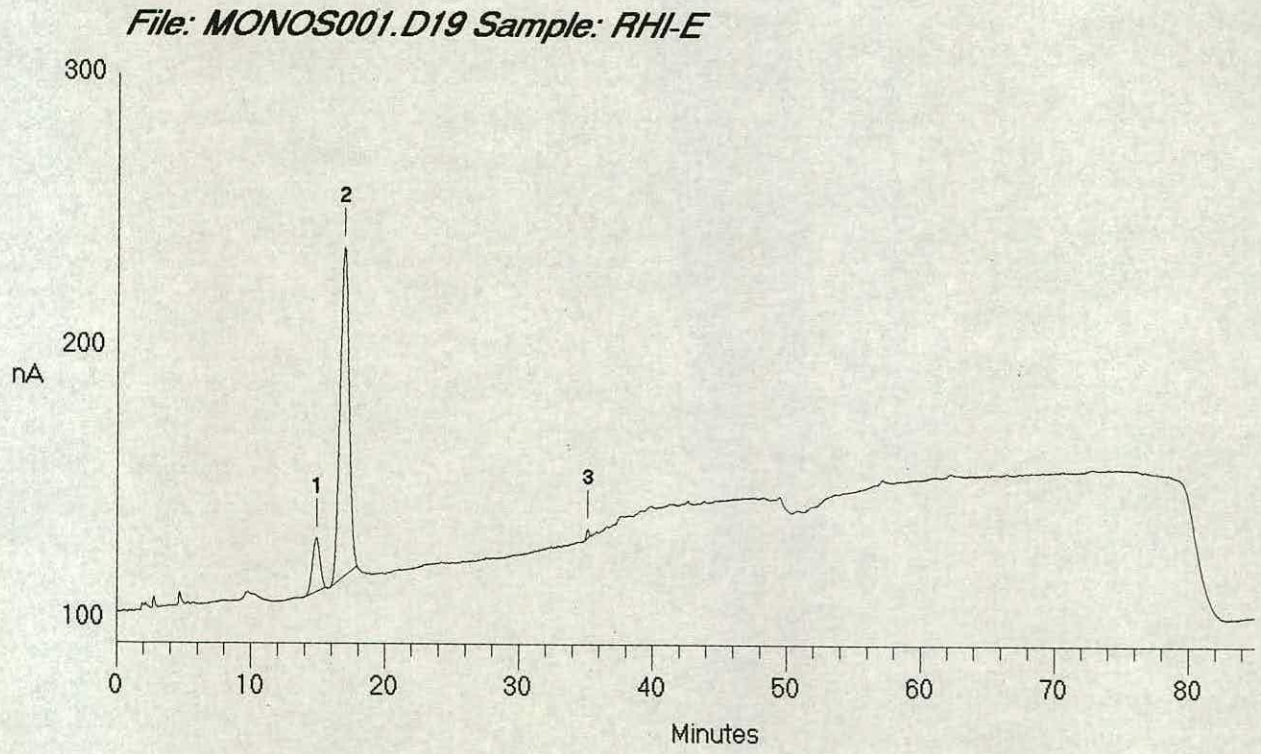


Figure 3.17. (continued)

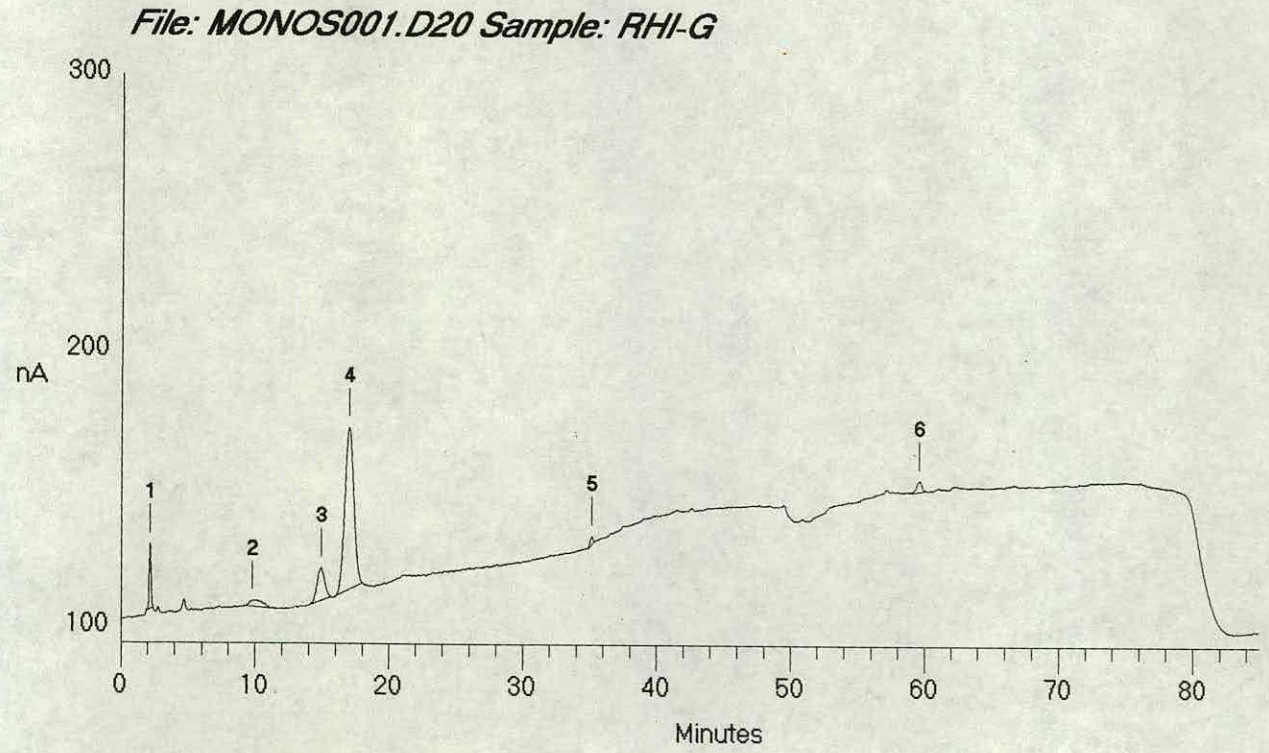


Figure 3.17. (continued)

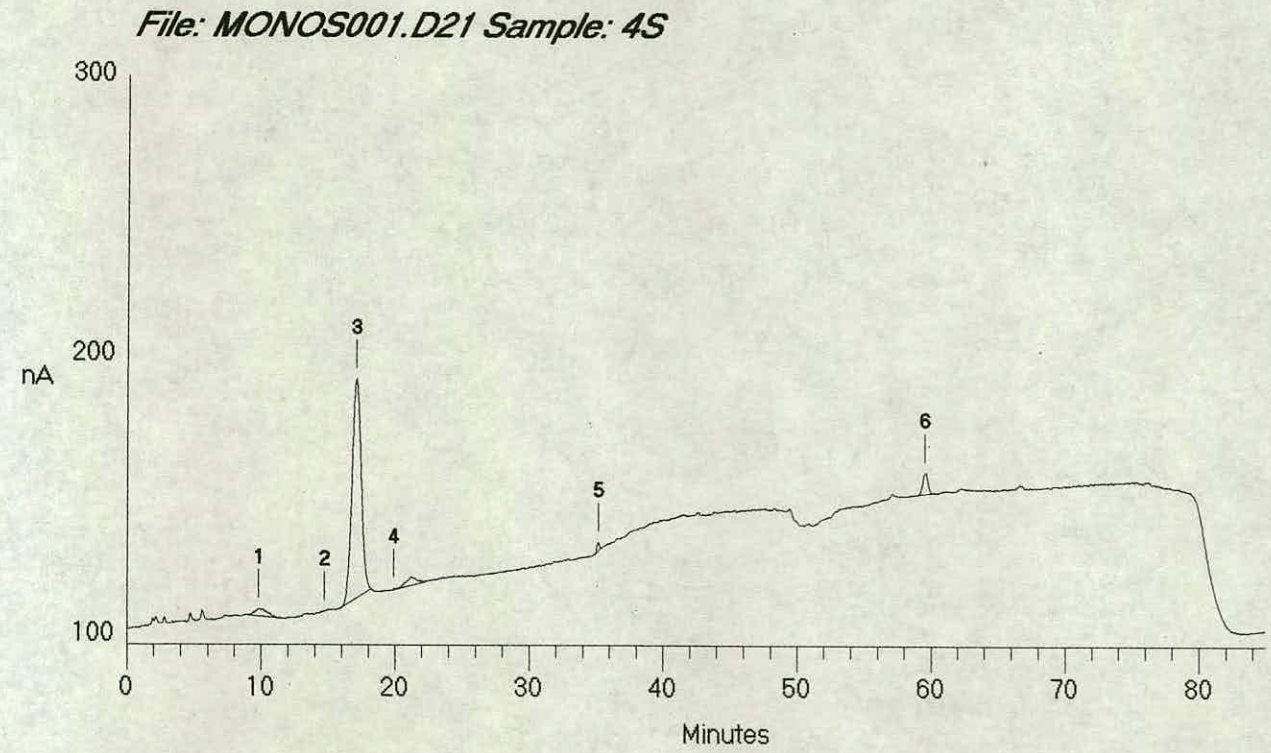


Figure 3.17. (continued)

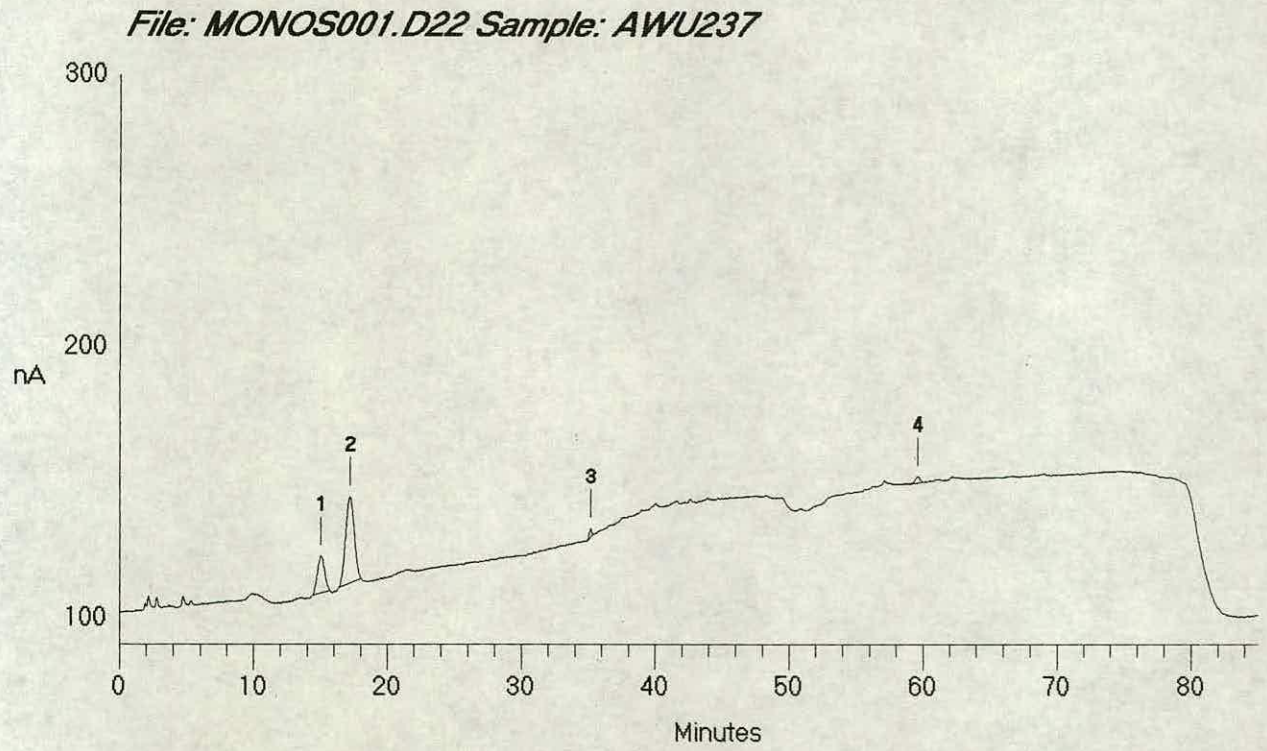


Figure 3.17. (continued)

Figure 3.17. (continued)

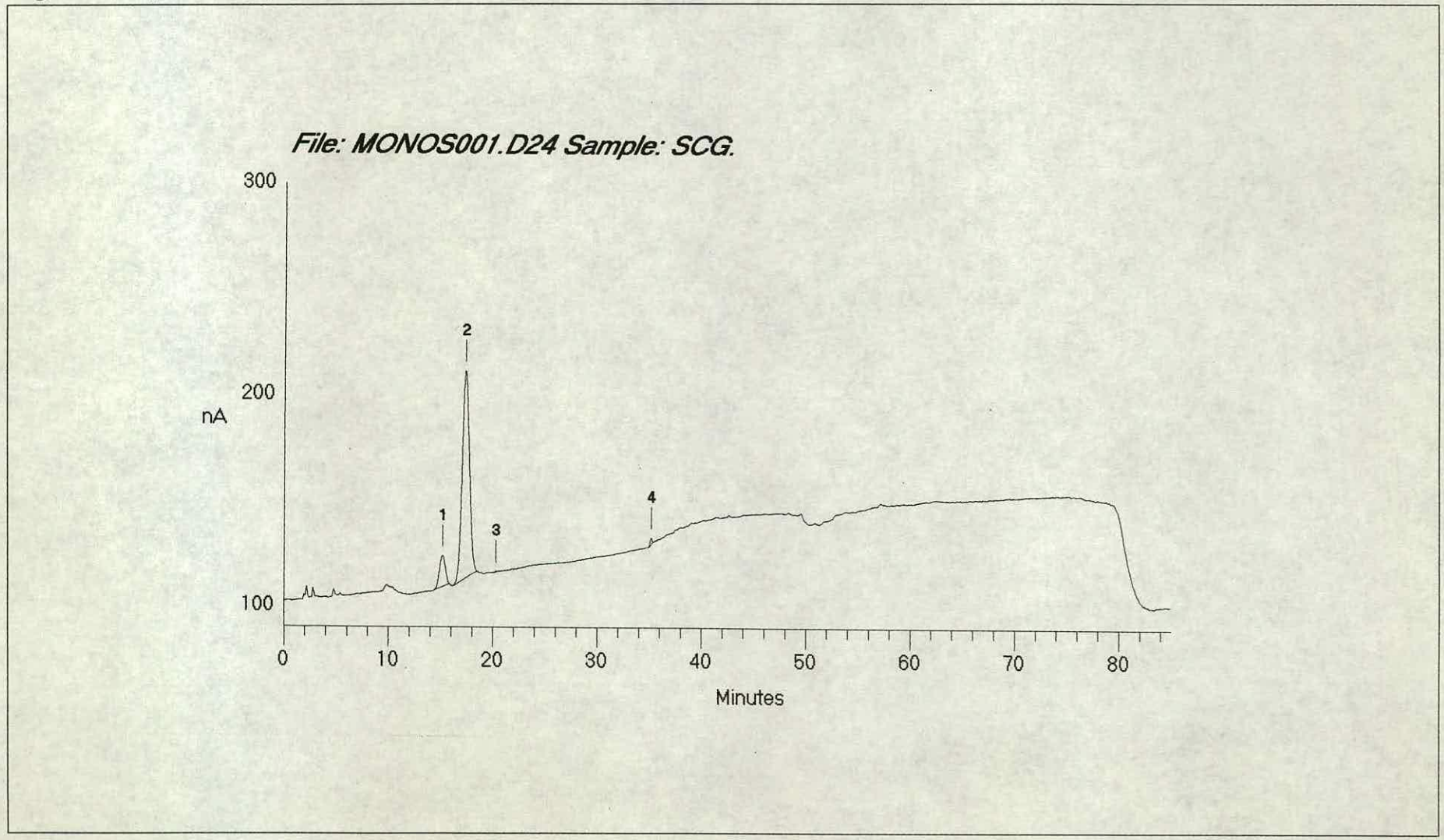


Table 3.13. The relative amounts of the constituent compounds of the carbohydrate component of the exopolysaccharides (based on HPLC -Dionex- analysis)

EPS	Ratio of Rha : Fuc : Man : Glc : Gal : GlcA : GalA	Possible stoichiometric ratio
SL2a	2.5 : 2.7 : 3.4 : 6.0 : 18.6 : 1 : 0	Man 1 : Glc 2 : Gal 6 Rha*, Fuc*
SL2b	0 : 0 : 0 : 3.3 : 1 : 0 : 0	Glc 3 : Gal 1
SL3	5.8 : 0 : 0 : 85.5 : 34.1 : 0 : 0	Glc 5 : Gal 2 Rha*, GalA*
SL4	7.1 : 0 : 0 : 92.1 : 27.5 : 0 : 1	Glc 3 : Gal 1 Rha*, GalA*
SL5	1 : 0 : 0 : 10 : 3.1 : 0 : 0	Glc 3 : Gal 1 Rha*
SL7b	0 : 0 : 0 : 8.4 : 1 : 0 : 0	Glc 8 : Gal 1
SL7c	0 : 0 : 1 : 1.9 : 4.5 : 0 : 0	Man 1 : Glc 2 : Gal 4
SL9a	1 : 0 : 0 : 18.4 : 5.6 : 0 : 0	Glc 3 : Gal 1 Rha*
SL9b	1.5 : 0 : 1 : 26.6 : 3.6 : 0 : 0	Glc 7 : Gal 1 Rha*, Man*
SL10	1 : 0 : 0 : 11.2 : 5.5 : 0 : 0	Glc 2 : Gal 1 Rha*
SL11a	1 : 0 : 3.6 : 8.4 : 1.1 : 0 : 0	Man 1 : Glc 2 Rha*, Gal*
SL12	1 : 0 : 0 : 15.6 : 5.9 : 0 : 0	Glc 5 : Gal 2 Rha*
Rhi-A	0 : 0 : 0 : 23.8 : 3.8 : 1 : 0	Glc 6 : Gal 1 GlcA*
Rhi-E	0 : 0 : 0 : 7.3 : 1 : 0 : 0	Glc 7 : Gal 1
Rhi-G	1.5 : 0 : 0 : 25 : 4.2 : 1 : 0	Glc 6 : Gal 1 Rha*, GlcA*
AWU237	0 : 0 : 0 : 25.1 : 8.7 : 1 : 0	Glc 6 : Gal 2
H_3	1 : 0 : 0 : 10 : 0 : 0 : 0	Glc 1, Rha*
4S	1 : 0 : 0 : 21.3 : 0 : 1.1 : 0	Glc 1, Rha*, GlcA*
Scg.	0 : 0 : 0 : 8.4 : 1 : 0 : 0	Glc 8 : Gal 1

Rha= Rhamnose, Fuc= Fucose, Man= Mannose
Glc= Glucose, Gal= Galactose, Scg= Succinoglycan
GlcA= Glucuronic acid, GalA= Galacturonic Acid
* = trace amounts

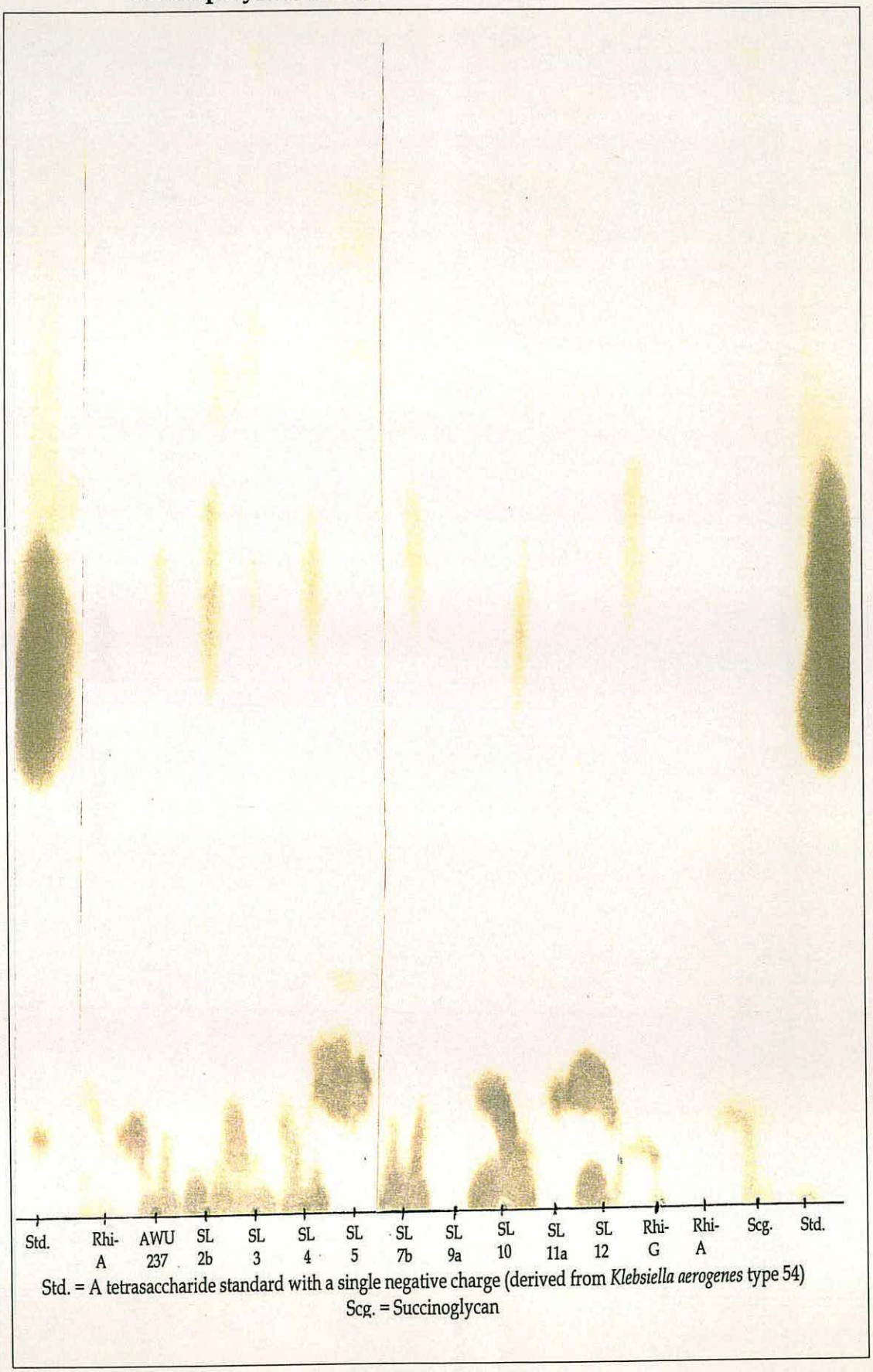
about 71% structural similarities to succinoglycan. It should be noted that there could be exceptions for a direct relationship between the succinoglycan-type structure and the susceptibility. For instance, a polymer which is similar to succinoglycan but possesses some additional substituent group(s) that cause a steric-hindrance would not exhibit a high degree of susceptibility to the depolymerase enzyme.

Table 3.14. Action of succinoglycan depolymerase on rhizobial exopolysaccharides

Substrate polysaccharide	Degree of activity (%)
Succinoglycan	100
SL2a	13.4
SL2b	88.4
SL3	90.0
SL4	92.8
SL5	90.5
SL7b	94.5
SL7c	20.5
SL9a	90.3
SL9b	94.3
SL10	93.7
SL11a	91.3
SL12x	93.9
Rhi-A	29.7
Rhi-E	97.7
Rhi-G	33.6
4S	37.2
AWU237	70.8
Rhizobium meliloti, IFO 13336	46.3

The breakdown products of exopolysaccharides by the succinoglycan depolymerase preparation were analysed by paper electrophoresis. The movement of oligosaccharides on the electrophoresis paper, which is determined by the charge and size of the molecules, are shown in figure 3.18. A tetrasaccharide with a single negative charge (derived from *Klebsiella aerogenes*- type 54) was used as the standard. Oligosaccharide fragments with negatively charged components such as uronic acids or succinate substituent groups were the molecules expected to travel longer distances on the electrophoresis paper. The hydrolysates of SL3, SL5, SL9a, SL10 and SL12x showed prominent spots of oligosaccharides on the electrophoresis paper which travelled the same distance as the standard and therefore could be tetrasaccharides produced by the action of succinoglycan depolymerase. This was possibly evidence for the presence of uronic acids, pyruvate and succinate groups. SL2b and SL4 also showed spots on the same area but in a low intensity indicating a lesser hydrolysis into that level of oligomers. The normal substrate for the enzyme, succinoglycan, had an unidentified spot well ahead of the tetrasaccharide standard. SL4 also showed a spot at the same distance, indicating that it might be a succinoglycan.

Figure 3.18. Paper electrograms of the products of succinoglycanase action on exopolysaccharides



Std. = A tetrasaccharide standard with a single negative charge (derived from *Klebsiella aerogenes* type 54)
Scg. = Succinoglycan

Autohydrolysis of exopolysacchrides

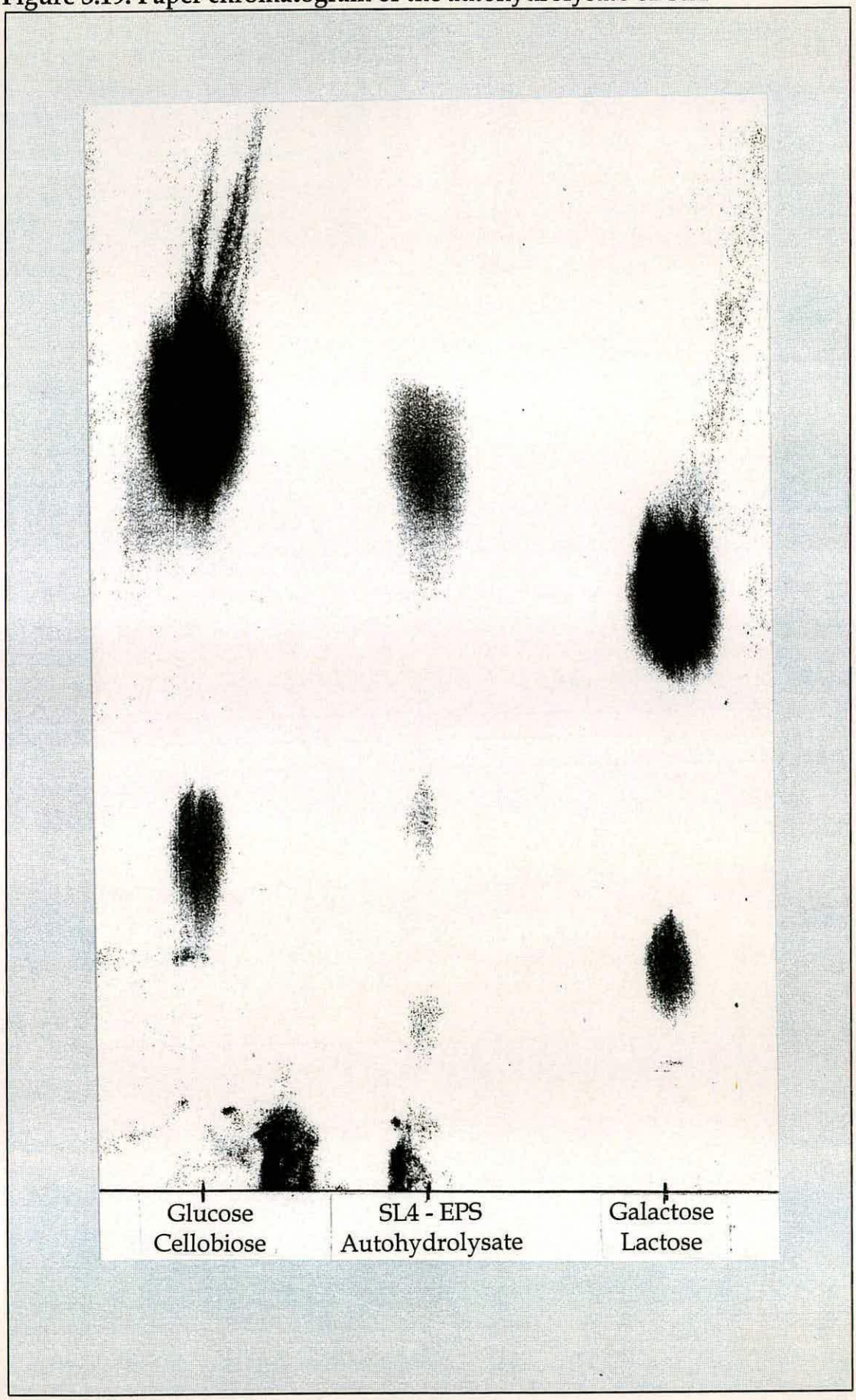
A concentrated solution of the exopolysaccharide of SL4, which, from other evidence, was known to carry acidic components, was left overnight for a possible auto-degradation due to the intrinsic acidity. The autohydrolysate, which was analysed by paper chromatography (figure 3.19), showed the presence of breakdown products confirming the acidic nature of the polymer. Glucose was prominent on the chromatogram with two more spots representing larger oligosaccharides.

Susceptibility to endogenous enzymes

Cell lysates of the Sri Lankan strains, in which the enzyme activities like glycosidases were detected and estimated, were tested against their own exopolysaccharides. The results of these assays, which were obtained through thiobarbituric acid (TBA), glucose oxidase and reducing sugar tests on breakdown products, were interpreted here as to derive possible information on structure/composition of the exopolysaccharides.

It was found by the TBA test that no lyase (eliminase) activity, which cleaves certain activated glycosidic linkages (p. 71) of acidic heteropolysaccharides, was present in any of the cell lysate-exopolysaccharide assays. However, this

Figure 3.19. Paper chromatogram of the autohydrolysate of SL4

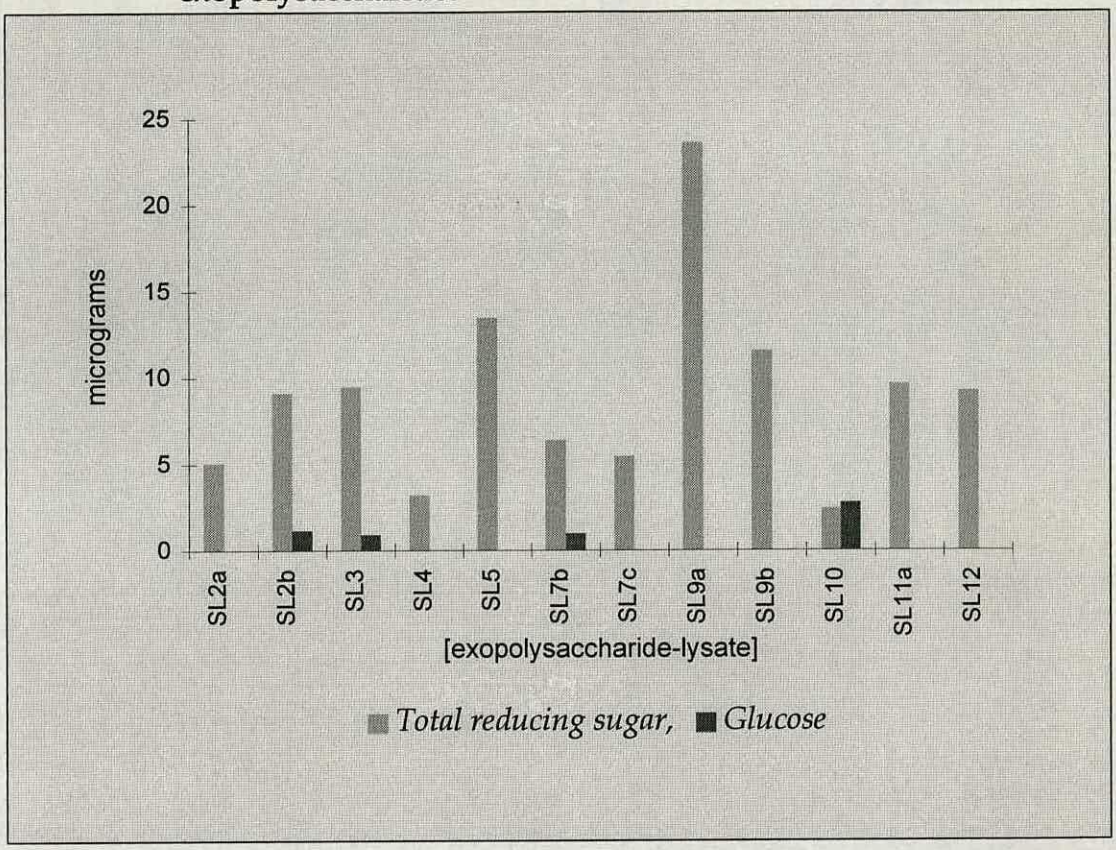


observation alone was not sufficient to conclude that the exopolysaccharides were of not that particular type, i.e., not similar to the acidic exopolysaccharide -succinoglycan- of *Rhizobium trifolli* (Hollingsworth, *et al.*, 1984), because, few of the succinoglycan-type polysaccharides possess uronic acid linkages susceptible to the action of lyases.

Figure 3.20. shows the amounts of total reducing sugar and glucose released from exopolysaccharides (μg mg^{-1} cell lysate protein) due to the hydrolytic enzyme activities of their own cell lysates. All the strains were capable of enzymatic release of reducing sugar from the polymers while the cell lysates of SL2b, SL3, SL7b and SL10 were able to hydrolyse their exopolysaccharides further to release glucose, although the extent of activities was very low.

From the previous results on glycosidase activities, it was concluded that among the strains which showed the ability to release reducing sugar, the strains SL2b, SL3, SL5, SL9a, SL9b, SL10 and SL11a possessed β-(1,4)- and β-(1,2)-glucosidases. It had also been found that SL3 and SL9b were α-glucosidase-positive to a significant level. SL9b showed a high degree of α-galactosidase activity as well. The release of reducing sugar, and, more specifically glucose, from exopolysaccharides may be due to one of these enzyme activities or due to the total activity of several of them.

Figure 3.20. Total reducing sugar and glucose (μg) released from exopolysaccharides



4. Conclusions

As pointed out earlier , the studies on the *Rhizobium*-legume symbiosis have mainly concentrated on temperate systems in which the nodules are of indeterminate type. Hirsch (1992) summarised the differences between indeterminate nodulation of temperate systems with determinate type of nodules found in tropical/ sub-tropical systems (p. 32). In addition to other differences it was pointed out that the exopolysaccharide mutants of rhizobia from determinate systems, unlike their temperate counterparts, were able to give rise to nitrogen-fixing nodules. One of the main aims of the present study was, therefore, to compare the exopolysaccharides of the Sri Lankan strains with the rhizobial strains of temperate origin.

Table 4.1 gives a list of components of a number of temperate rhizobial exopolysaccharides analysed in various research laboratories and tropical and temperate strains analysed in the present study. Although there are some differences between temperate and tropical strains it could be concluded that these differences were not very significant and nothing more than the differences between individual bacteria of each group. The carbohydrate components, i. e. the sugar compositions, as revealed by paper chromatography and HPLC studies, were remarkably similar in all but SL2a. This was confirmed by the resistance of this polysaccharide to enzyme

Table 4.1. Composition of different rhizobial exopolysaccharides`

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	Acetate	Succinate	References
R. phaseoli 127K36	5	1	-	-	-	-	2	-	2	?	?	Dudman *et. al.* (1983a)
R. phaseoli LPR49	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. trifolii NA30	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. trifolii 0403	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. trifolii LPR5	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. leguminosarum 128c53	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. leguminosarum 128c63	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. leguminosarum LPR1	5	1	-	-	-	-	2	-	2	?	?	McNeil *et. al.* (1986)
R. phaseoli 127K44	5	2	-	-	-	-	3	-	1	?	?	Franzen *et. al.* (1983)
R. phaseoli 127K87	5	1	-	-	-	-	3	-	1	?	?	Dudman *et. al.* (1983b)

...continued

Table 4.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
Cowpea rhizobia (Tropical)												
Strain MI-50A	2	1	1	-	-	-	-	-	-	-	-	
Strain M6-7B	1	1	3	-	-	-	-	-	-	-	-	
Strain IRC 253	1	1	3	-	-	-	-	-	-	-	-	Hollingsworth *et. al.* (1985)
Strain 513-B	-	2	3	-	-	-	-	-	-	-	-	
Strain Ez-Aesch	-	2	3	-	-	-	-	-	-	-	-	
Strain IRC291	-	-	1	-		-	-	-	-	-	-	
R. leguminosarum bv. *phaseoli* CFN42	5	1	-	-	-	-	2	-	2	1	?	Gil-Serrano *et al.* (1992)
R. meliloti 2021	7	1	-	-	-	-	-	-	1	?	?	Jansson *et. al.* (1977)
R. meliloti 2021 Inf^{-1} mutant	7	1	-	-	-	-	-	-	?	?	?	Muller *et. al.* (1988)
R. trifolii	5	1	-	-	-	-	1	-	?	?	?	Jansson *et. al.* (1979)
(Brady)R. japonicum 3I1B	2	1	1	-	-	-	-	1	?	2	?	Mort and Bauer (1982)

continued..

Table 4.1.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
R. trifolii BART A (Nod^{-1})	5	1	-	-	-	-	2	-	2	3	?	Sömme (1985)
R. meliloti Rm1021 (EPS I)	7	1	-	-	-	-	-	-	1	1	1	Chouly *et. al.* (1995)
R. meliloti Rm1021 (EPS II)	1	1	-	-	-	-	-	-	1	1	?	Her *et. al.* (1990)
R. trifolii 4S	5	-	-	-	-	-	2	-	1	1	?	Amemura *et. al.* (1983)
R.meliloti IFO 13336	5	1	-	-	-	*	1	-	?	?	?	Amemura *et. al.* (1981)
R. leguminosarum bv. *trifolii* RBL5529	5	1	-	-	-	-	2	2	?	?	?	van Workum *et al.* (1997)

continued..

Table 4.1.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
SL2a	2	6	1	-	-	-	-	-	2	0	1	
SL2b	3	1	-	-	-	-	-	-	0	5	1	
SL3	5	2	-	-	-	-	-	-	1	1	2	
SL4	3	1	-	-	-	-	-	-	0	3	1	
SL5	3	1	-	-	-	-	-	-	0	1	0	
SL7b	8	1	-	-	-	-	-	-	0	0	0	

continued..

Table 4.1.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
SL7c	2	4	1	-	-	-	-	-	0	0	0	
SL9a	3	1	-	-	-	-	-	-	0	1	0	
SL9b	7	1	-	-	-	-	-	-	0	0	0	
SL10	2	1	-	-	-	-	-	-	2	0	1	
SL11a	2	-	1	-	-	-	-	-	0	0	1	
SL12	5	2	-	-	-	-	-	-	3	2	5	

continued..

Table 4.1.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
Rhi-A	6	1	-	-	-	-	-	-	3	1	0	
Rhi-E	7	1	-	-	-	-	-	-	0	0	1	
Rhi-G	6	1	-	-	-	-	-	-	1	0	0	
AWU237	6	2	-	-	-	-	-	-	1	2	0	
H3	1	-	-	-	-	-	-	-	?	?	?	
4S	1	-	-	-	-	-	-	-	2	1	0	

continued..

Table 4.1.1. (*continued*)

Strain	Glucose	Galactose	Mannose	Fucose	Rhamnose	Other sugars	Glucuronic acid	Galacturonic acid	Pyruvate	O-acetyl	O-succinyl	References
Scg.	8	1	-	-	-	-	-	-	1	0	1	

* = Riburonic acid
? = The ratio was not mentioned

hydrolysis. SL7c also showed resistance to the succinoglycan depolymerase preparation.

The organic substituent groups, i. e. acetate, pyruvate and succinate were also in similar proportions although considerable differences existed between the different polysaccharides. Hollingsworth *et. al.* (1985) was the only publication found which showed significant differences in exopolysaccharide composition of several strains of cowpea rhizobia, which were of tropical origin (table 4.1). Those strains were devoid of any organic substituent and their carbohydrate compositions were also different by having, for example, high mannose content. However, our results do not support against any hypothesis which suggests significant differences between tropical and temperate rhizobial exopolysaccharides. The susceptibility to the succinoglycan depolymerase enzyme, to which most of the Sri Lankan strains were highly susceptible, also confirmed that these polymers were basically of succinoglycan-type. They thus appear to be mainly composed of octasaccharide repeat units with the majority containing D-glucose and D-galactose where, in the molar ratio, the former was several times more than the latter. Strains SL2a and SL7c appear to markedly different from others. The presence of rhamnose traces in most of the exopolysaccharides was also not common.

When the composition of temperate and tropical rhizobial exopolysaccharides is concerned, it can also be concluded that it is not the

basic composition of the molecules what determine the specific involvement of exopolysaccharides in symbiosis. The crucial determinant factor of expolysaccharides might, if not the composition, be the specific three dimensional arrangement of the molecules.

Although almost all the rhizobial strains used in this study possessed strong enzyme activities such as glycosidase action, none of the bacteria were able to hydrolyse any plant structural polymer. Hence, those enzyme activities could be attributed mostly to metabolic requirements of the rhizobia. The ability showed by some strains to depolymerise their own exopoly-saccharides is significant, especially in the context that some authors suggested a role played by rhizobial enzymes in the breakdown of their exopolysaccharides, either to utilise the end-products in some stage of nodulation or, simply to remove them if they hinder signal communication between the bacteria and the host plant. However, this activity is neither strong nor widespread as Dunn and Karr (1992) reported. The amylase activity showed by two of the Sri Lanka strains has only recently been reported for a small number of rhizobia. It was found in the present study that both cell interior and the extracellular fractions of both strains were hydrolysing starch *via* large oligosaccharides to glucose, the basic unit. This may be the result of having either several different amylases or amylases plus glycosidases. The extracellular amylases of both strains were found to

be strongly inducible. The starch present in the host cells may, hence, be utilised by these particular strains.

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